Photochemical Protection of Riboflavin and Tetrapyrroles with Light Scattering Technology

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ABSTRACT

The effectiveness of titanium dioxide (TiO_2) in polyethylene films at preventing the photooxidation of riboflavin in a model solution was evaluated. Five different TiO_2 loads (0.5-8.0 wt%), each at 3 different thicknesses (50-100 um) were evaluated. A photochemical reactor, equipped with a 350W mercury lamp, provided full spectrum light or narrow bandwidth wavelength exposure, using filters allowing transmission at 25 nm wavebands at maximum peak height at 450, 550, or 650 nm. Riboflavin concentration was measured by HPLC over 8 hours of exposure. Increased TiO_2 load and thickness significantly affected riboflavin photooxidation ($p<0.05$). TiO_2 load had more influence on protection provided to riboflavin than did film thickness. Film opacity correlated linearly with decreased photooxidation (R^2 of 0.831 & 0.783 for full spectrum and 450 nm bandpass-filter sets, respectively). Riboflavin photooxidation proceeded most rapidly with the full spectrum exposure (light intensity 118 ± 17.3 mW). Photooxidation occurred in the 450 nm bandpass-filter, but not for 550 & 650 nm sets (light intensities of 2.84 ±0.416, 3.36 ±0.710, and 0.553 ± 0.246 mW, respectively). Effect of fluorescent light-exposure (2020-1690 lux) on the same system was monitored over 2 days. Riboflavin degradation in the photoreactor proceeded ~300 times faster than under fluorescent lighting. Riboflavin degradation was found to significantly increase with the addition of chlorophyll-like tetrapyrroles ($p<0.05$). Riboflavin was found to significantly

decrease the degradation rate of the tetrapyrroles pyropheophytin *a* and pheophytin *a* ($p<0.05$). The decrease in rate was not significant for chlorophyll *a* ($p>0.05$).

ACKNOWLEDGEMENTS

During my first contact with the Virginia Tech Department of Food Science and Technology (FST), Dr. Joseph Marcy told me that it would be easier to explain what food science is in terms of what it is not than what it is. During these past few years, I have often found that sentiment to be very accurate, not to mention useful when describing food science to curious family and friends. I would like to thank my advisor, Dr. Susan Duncan for her guidance and support during my time at FST and also for my scholastic introduction to food science.

Thank you to my committee members, Dr. Sean O'Keefe and Dr. Janet Webster for your advice and encouragement during my stay here at Virginia Tech. Many others have also provided invaluable help in many ways. Thank you Dr. Sabrina Hannah, Harriet Williams, Walter Hartman, Joe Boling, Kim Waterman, and Dr. Wang. Thank you Dr. Andrew Neilson and Katie Goodrich for your help these last few days.

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CHAPTER I

Introduction

It has been well established since the 1950s that light exposure in dairy products leads to nutrient loss and off-flavors *(1–3)*. This process, called photooxidation, is of particular concern in milk because the oxidation occurs quickly under the fluorescent lights used in store display cases, resulting in perceptible negative changes in flavor within hours *(4)*. Photosensitizers are key compounds in this process because their presence leads to the destruction of compounds that are otherwise unaffected by light *(5,6)*. Riboflavin has long been viewed as the photosensitizer of importance in milk *(3)*. Recent research has suggested that chlorophyll and chlorophyll-like compounds may also act as significant photosensitizers in milk *(7–13)*. Many companies avoid packaging milk in opaque containers, which may provide protection against photooxidation, because consumers favor materials that allow them to see the product *(11,12)*. In addition, even visually opaque materials are not always totally effective at protecting against photosensitizer excitation. We propose that packaging that only blocks those wavelengths that excite the photosensitizers would provide an excellent compromise between protecting dairy product quality and marketing dairy products. The wavelengths at which chlorophyll and riboflavin undergo photooxidation do have some overlap *(6,12)*. More significantly, chlorophyll will undergo photooxidation at higher visible wavelengths than riboflavin will. Knowing more about the impact chlorophyll has on photooxidation will be helpful when making packaging decisions for dairy products with various amounts of chlorophyll, such as milk and cheeses.

The effectiveness of low density polyethylene (LDPE) films of varying thickness and titanium dioxide loads in preventing riboflavin degradation in a photoreactor was determined. This project investigated how riboflavin and chlorophyll interact during light exposure by measuring their oxidative degradation in a model system. These investigations also allowed for comparison of the photooxidative rates of riboflavin under retail conditions and in a photoreactor.

The photoreactor can be used to significantly lower the time to test experimental light treatments, but how it compares to fluorescent dairy-case lighting is currently unknown. This knowledge would be of particular interest to companies that have funded other photodegradation research at Virginia Tech and future researchers here and elsewhere as well. If the relationship between degradation in the photoreactor and conventional retail degradation was know with certainty, future investigators could accomplish in a matter of hours what would otherwise take weeks.

Quantifying the effectiveness of the two parameters investigated (low density polyethylene film thickness and weight percentage of TiO_2) as it pertains to riboflavin degradation will allow more efficient development and identify valuable applications of protective packaging material. Hopefully, this will result in lower amounts of light oxidized milk reaching the consumer and at lower costs to all parties. This would benefit consumers, packagers and producers of dairy products.

In addition, chlorophylls *a & b* (along with other chlorins and porphyrins) have only recently been implicated as photosensitizers in dairy products *(7)*. It is still unknown what impact, if any, the presence or absence of these photosensitizers have in

concert with riboflavin. This research serves as a beginning step, using a model system, in understanding the interactions of photosensitizers within food systems.

Goals and Objectives

Goal: To characterize photoreaction rates of riboflavin and chlorophyll-like compounds for use in improving packaging materials that protect light sensitive foods.

Photoreactor study: Protection of riboflavin from light by low density polyethylene opacity, based on film thickness and titanium dioxide (TiO_2) load.

Objective 1-1: Quantify rates of riboflavin degradation, in a phosphate buffer maintained at 4°C, under full light and select visible wavelength regions (25 nm half-height-full-width bandwidth; peak wavelengths at 450, 550, 650 nm) using a photochemical reactor over relevant periods, up to 8 hrs.

Objective 1-2: Quantify change in riboflavin degradation rates, in phosphate buffer maintained at ~4°C, under full light and select visible wavelength regions (25 nm bandwidth; peak wavelengths at 450, 550, 650 nm) using a photochemical reactor over relevant periods of light exposure, as a function of LDPE films with differing thicknesses and TiO_2 loads.

Storage Study Part A: Estimate the relationship of light conditions (light intensity, time duration) in the photochemical reactor to refrigerated dairy case for riboflavin degradation in fluid milk model system.

Objective 2-1: Quantify riboflavin degradation rate, in phosphate buffer maintained at 6 ±1 °C, stored under fluorescent lights over a 2 day shelf-life.

Objective 2-2: Develop a predictive equation for riboflavin degradation, in a phosphate buffer, over time at 4°C, by comparing rates of riboflavin degradation under refrigerated (6 ± 1 °C) dairy case conditions (fluorescent lighting) over 2 days of light exposure and photochemical reactor conditions.

Storage Study Part B: Characterize the contribution of photosensitizers (tetrapyrroles, riboflavin) on photooxidation.

Objective 3-1: Quantify degradation rate of riboflavin, in a phosphate buffer & acetone system at maintained at 6 ± 1 °C and stored under fluorescent lights over a 2 day shelf-life.

Objective 3-2: Quantify degradation rates of tetrapyrroles (chlorophyll *a,* pyropheophytin *a,* and phoephytin *a*), when present in a phosphate buffer & acetone system maintained at 6 ± 1 °C and stored under fluorescent lights over a 2 day shelf-life.

Objective 3-3: Quantify degradation rates of tetrapyrroles (chlorophyll *a,* pyropheophytin *a,* phoephytin *a*) and riboflavin in a phosphate buffer & acetone system maintained at 6 ± 1 °C and stored under fluorescent lights over a 2 day shelf-life.

CHAPTER II

Literature Review

Light As a Source of Chemical Energy

The photons in visible and ultraviolet (UV) light, like all parts of the electromagnetic spectrum, contain different quanta (precise amounts) of energy in a direct proportion to their wavelength. Mathematically, this is defined by the equation $E=hc/\lambda$, where E is energy, h is Planck's constant and λ is the wavelength of the photon. Therefore, when a precise amount of energy must be imparted to a system such as a photosensitive compound (or chromophore), only light with an equally precise wavelength will be capable of doing so *(14)*. This is of particular importance for smaller molecules that have very discreet energy levels, but less so for molecules that have aromatic rings or other sources of delocalized molecular orbitals. Given the large number of compounds found in food, only a small number are capable of absorbing light in the UV-Vis spectrum. Those that do and then proceed to transfer this energy to other molecules are known as photosensitizers. Photosensitizers have the unusual capability of undergoing intersystem crossing from the first (lowest) singlet excited state (brought about by the increase in energy from a photon) to the first excited triplet state *(15)*. For example, riboflavin can accept the energy from a photon to promote an electron to a higher energy orbital without changing the spin multiplicity of the molecule, and then can undergo intersystem crossing (flipping the spin of the electron), which does change the spin multiplicity *(12,15,16)*.

$^1\text{Rib} + \Upsilon \rightarrow {}^1\text{Rib}^*$ Excitation by a photon (can occur in photosensitizers and non-photosensitizers)

$^1Rib^* \rightarrow {}^3Rib^*$ Intersystem crossing (a key property of photosensitizers) *(15)*

The singlet and triplet states are named based upon the spin multiplicity of the molecule *(14–16)*.

Type I and Type II Photoreactions

Photosensitizers can pass the energy accepted from a photon on to other photo-labile compounds either directly or by using oxygen as an intermediary *(5,6,12,17)*. When the interaction occurs directly, it is denoted as a Type I photoreaction. When the first interaction leads to the development of singlet oxygen ($^1O_2$) from ground state triplet oxygen ($^3O_2$), it is denoted as Type II. The seemingly simple distinction between these two types is complicated by oxygen's involvement in both of these categories, as a Type I reaction eventually produces a superoxide radical ($^1O_2^{\bullet-}$). Kristensen and others (2002) illustrated the Type I and Type II mechanisms of photooxidation of riboflavin in depth *(18)*.

In Type I photooxidation of riboflavin, excited triplet state riboflavin (^{3}Rib*) interacts with a photo-labile substrate (Sub) resulting in a radical compound ($Sub^{\bullet+}$) and reduced riboflavin ($^2Rib^{*-}$). Reduced riboflavin can then return to the ground state and proceed to form the radical superoxide anion ($O_2^{\bullet-}$) from ground state oxygen. This superoxide anion can begin a cascade of free radical reactions oxidizing many compounds. It is also important to note that this cycle regenerates the ground state riboflavin that is then free to accept energy from a photon of appropriate wavelength, beginning the cycle anew.

The Type II mechanism begins with the excitation of ground state riboflavin by light, identical to the start of Type I. As in Type I, the next step is intersystem crossing

from the initial singlet state to riboflavin excited triplet state ($^3Rib^*$). It is the fate of $^3Rib^*$ where the Type I and Type II mechanisms diverge. Instead of reacting directly with a substrate, high energy singlet oxygen ($^1O_2$) is produced from the transfer of energy from $^3Rib^*$ to molecular oxygen. The Type II mechanism also regenerates ground state riboflavin just like Type I does.

Riboflavin

Riboflavin is an orange-yellow photosensitizer of great importance in dairy photooxidation *(1–3,5–7,9,10,12,13)*. In addition to appearing in bovine dairy products, riboflavin in an essential vitamin in humans (designated as vitamin B2) *(6)*. Lack of riboflavin can interfere with numerous processes in the body including metabolism, hormone production, red blood cell production and the functioning of many organs such as the skin, eyes, mucous membranes and brain *(6)*. Riboflavin also has a role in metabolism during the electron-transport system appearing as a part of the coenzymes flavin mononucleotide and flavin adenine dinucleotide *(6)*. As discussed earlier, the many delocalized double bonds in riboflavin is responsible for riboflavin's action as a photosensitizer *(4)*.

Chlorophyll, Porphyrins and Tetrapyrroles

Chlorophylls, tetrapyrroles and other porphyrins and chlorins have recently been implicated as photosensitizers of milk and dairy products. Most of the work in discovering this fact was done by research led by Wold as well as by Webster, Duncan and others *(7–13)*. One of the consequences of this finding is that it is now clear that, in order to protect against photooxidation, milk must be protected from wavelengths above

those which excite riboflavin *(7–10,12,13)*. Riboflavin's main absorption wavelengths are at approximately 225, 270, 370, 400, 450, and 570 nm at neutral pH *(6,12)*. Chlorophyll *a* absorbs throughout the UV visible spectrum with maximums at 410, 430, and 662 nm *(12)*. Chlorophyll *b* similarly absorbs at 453 and 642 nm *(12)*. Several additional species have been cautiously put forth by investigators as being responsible for photooxidation occurring at wavelengths above 500 nm. Compounds that may be of concern in photooxidation of milk are protoporphyrin, hematoporphyrin, chlorophyll *a,* chlorophyll *b,* and potentially other chlorins and porphyrins *(7,9,10)*. These results have been obtained through a combination of fluorescence spectroscopy, sensory analysis and the use of colored filters during light exposure *(7–13)*.

As well as being an essential component in photosynthesis, the photoactive properties of chlorophyll *a* are being explored for use in the treatment of human leukemia *(19)*. The work of Dentuto and others (2007) suggests that introducing chlorophyll *a* while dissolved in ethanol is a possible method of achieving chlorophyll micelles in an aqueous system. Aggregated chlorophyll *a* appears to undergo far less type II photosensitization (singlet oxygen production), likely because of energy transfers among the aggregated molecules *(19)*.

Packaging

Light is known to be responsible for specific defects in milk flavor and nutrition, and the implications of light on dairy products has been reviewed by Singh et al *(1)* and Allen and Parks *(3)*. The simplest solution to this is to package the milk in such a way that prevents all light transmission. This is both possible and cost effective. The barrier to this solution is both cost and consumers' desire to see the food product they are

purchasing *(12)*. As a compromise, recent research has focused on blocking only those wavelengths necessary and most effective at preventing milk and dairy product photooxidation *(5,8,10–13)*. The goal is an effective compromise that allows the buyer to see the product with maximum detail and minimal color distortion. The relatively recent emphasis on photosensitizers in addition to riboflavin has highlighted the importance of determining which specific wavelengths of light are responsible for photodegradation in milk *(7–10,12,13,20)*.

Riboflavin's main absorption wavelengths are at approximately 225, 270, 370, 400, 450, and 570 nm at neutral pH *(6,12)*. Blocking light in the UV range (200-400 nm), as accomplished by adding UV absorbers into polyethylene terephthalate, allows for clear packaging and has been extensively studied in milk products *(10–13,21)*. However, this range does not provide complete protection of riboflavin or milk flavor *(21)*.

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CHAPTER III

Photochemical Protection of Riboflavin and Tetrapyrroles with Light Scattering Technology

INTRODUCTION

Riboflavin is one of several photosensitizers occurring naturally in milk products *(1–6)*. Photooxidation of riboflavin leads to the degradation of other compounds in milk, contributing to the deterioration of both flavor and nutritional quality *(7–11)*. Riboflavin has been the primary focus of most research on milk and dairy product photosensitizers. Protection of riboflavin and milk quality from the effects of light can be addressed by designing packaging that reduces the amount of light energy that can interact with riboflavin or other photosensitizers *(3–5)*.

Recently, the importance of additional photosensitizers, tentatively identified as chlorophyll and other tetrapyrroles in dairy products, especially in cheese, has been a subject of research *(1,2,5,6,12)*. The wavelengths at which chlorophyll and riboflavin undergo photooxidation have some overlap *(3,6,8,9)*. Riboflavin's main absorption wavelengths are at approximately 225, 270, 370, 400, 450, and 570 nm at neutral pH *(8,9)*. Chlorophyll *a* absorbs throughout the UV visible spectrum with maximums at 410, 430, and 662 nm *(3)*. Chlorophyll *b* similarly absorbs at 453 and 642 nm *(3)*. Most significantly, chlorophylls and other tetrapyrroles will undergo photooxidation at higher visible wavelengths (642 and 662 nm for chlorophylls *a & b*) than riboflavin will. It is still unknown what impact, if any, the presence or absence of these photosensitizers may have when in concert with riboflavin. Recent studies of milk packaging have found that photooxidation is not completely inhibited by blocking riboflavin's excitation wavelengths (UV to ~550 nm) *(3–6)*. Knowing more about the impact that tetrapyrroles and chlorophylls have on photooxidation will be helpful when making packaging

decisions for dairy products, such as milk and cheeses, with various amounts of chlorophyll.

The effectiveness of polyethylene terephthalate films of varying thickness and titanium dioxide loads in preventing riboflavin degradation in a photoreactor was determined. A storage study investigated how riboflavin and select tetrapyrroles interact during light exposure by measuring their rates of photooxidation in a simple model system. The above research enabled an investigation of how the photooxidative rates of riboflavin under retail conditions and in a photoreactor compare.

Goals and Objectives

Goal: To characterize photoreaction rates of riboflavin and chlorophyll-like compounds for use in improving packaging materials that protect light sensitive foods.

Photoreactor study: Protection of riboflavin from light by low density polyethylene opacity based on film thickness and titanium dioxide (TiO_2) load.

Objective 1-1: Quantify rates of riboflavin degradation, in a phosphate buffer maintained at 4°C, under full light and select visible wavelength regions (25 nm half-height-full-width bandwidth; peak wavelengths at 450, 550, 650 nm) using a photochemical reactor over relevant periods, up to 8 hrs.

Objective 1-2: Quantify change in riboflavin degradation rates, in phosphate buffer maintained at ~4°C, under full light and select visible wavelength regions (25 nm bandwidth; peak wavelengths at 450, 550, 650 nm) using a photochemical reactor over relevant periods of light exposure, as a function of LDPE films with differing thicknesses and TiO_2 loads.

Storage Study Part A: Estimate the relationship of light conditions (light intensity, time duration) in the photochemical reactor to refrigerated dairy case for riboflavin degradation in fluid milk model system.

Objective 2-1: Quantify riboflavin degradation rate, in phosphate buffer maintained at 6 ±1 °C, stored under fluorescent lights over a 2 day shelf-life.

Objective 2-2: Develop a predictive equation for riboflavin degradation, in a phosphate buffer, over time at 4°C, by comparing rates of riboflavin degradation under refrigerated (6 ± 1 °C) dairy case conditions (fluorescent lighting) over 2 days of light exposure and photochemical reactor conditions.

Storage Study Part B: Characterize the contribution of photosensitizers (tetrapyrroles, riboflavin) on photooxidation.

Objective 3-1: Quantify degradation rate of riboflavin, in a phosphate buffer & acetone system maintained at 6 ± 1 °C and stored under fluorescent lights over a 2 day shelf-life.

Objective 3-2: Quantify degradation rates of tetrapyrroles (chlorophyll *a,* pyropheophytin *a,* and phoephytin *a*), when present in a phosphate buffer & acetone system maintained at 6 ± 1 °C and stored under fluorescent lights over a 2 day shelf-life.

Objective 3-3: Quantify degradation rates of tetrapyrroles (chlorophyll *a,* pyropheophytin *a,* phoephytin *a*) and riboflavin in a phosphate buffer & acetone system maintained at 6 ± 1 °C and stored under fluorescent lights over a 2 day shelf-life.

MATERIALS AND METHODS

1x Phosphate Buffer for Photoreactor Experiment. Riboflavin (purity≥99%, Sigma-Aldrich, St. Louis, MO) was dissolved in a phosphate buffer solution designed to provide a buffered pH and ionic strength similar to that of milk. The normal, or 1x strength buffer was composed of 4.94 g $NaH_2PO_4*H_2O$ (Sigma-Aldrich, St. Louis, MO), 2.02 g Na_2HPO_4 (Sigma-Aldrich, St. Louis, MO), and 0.09 g NaCl (Sigma-Aldrich, St. Louis, MO) dissolved in dH_2O to 1L. Buffer pH was 6.4-6.5 with a calculated ionic strength of I=0.08. The phosphate buffer was filtered using a Millipore filter apparatus with a 0.2 μm nylon filter. All of the phosphate buffer used in the photoreactor experiment was prepared as 1x phosphate buffer and was not diluted any further.

Riboflavin Stock for Photoreactor Experiment. The final stock solution concentration was 30 ppm riboflavin. Relative humidity from seasonal variation affected mass balance and was accounted for in the preparation of the stock solution. Stock solution preparation was done under low light conditions using a volumetric flask. The stock solution was placed in a dark cabinet for a minimum of 20 minutes with intermittent agitation by hand. The riboflavin stock was stored in a refrigerator (~5°C) and replaced weekly.

1.1x Phosphate Buffer for Storage Study Experiment. As before, riboflavin (purity≥99%, Sigma-Aldrich, St. Louis, MO) or tetrapyrroles were buffered by a phosphate buffer solution designed to provide a pH and ionic strength similar to that of milk during the experiment. Because the buffer was diluted with acetone or ddH_2O during the preparation of the experimental treatments, the initial concentration was

increased. The 1.1x buffer was composed of 5.43 g $NaH_2PO_4*H_2O$ (Sigma-Aldrich, St. Louis, MO), 2.22 g Na_2HPO_4 (Sigma-Aldrich, St. Louis, MO), and 0.099 g NaCl (Sigma-Aldrich, St. Louis, MO) dissolved in dH_2O to 1L. The pH of the 1.1x buffer prior to dilution was 6.40-6.47. The phosphate buffer was filtered using a Millipore filter apparatus with a 0.2 μm nylon filter. The 1.1x phosphate buffer that was for use in experimental mixtures not containing riboflavin did not have any riboflavin added.

Riboflavin Stock for Storage Study Experiment. The initial stock solution concentration was 33.3 ppm riboflavin in 1.1x strength phosphate buffer. Later, this was diluted using acetone or ddH_2O to a final concentration of 30 ppm riboflavin in 1x phosphate buffer. Stock solution preparation was done under low light conditions using a volumetric flask and 1.1x phosphate buffer. The stock solution was placed in a dark cabinet for a minimum of 20 minutes with intermittent agitation by hand. The riboflavin stock was prepared individually for each replication.

Tetrapyrrole Stock for Storage Study Experiment. The initial stock solution concentration of tetrapyrrole stock was made by dissolving ~95 mg of chlorophyll oil (MP Biolmedicals, Solon, OH; Cat # 206208, Lot# 6354K) in ~65 g HPLC grade acetone (Sigma-Aldrich, St. Louis, MO). The primary chlorophyll-like components (by abundance) of the tetrapyrrole mixture were chlorophyll *a,* pyropheophytin *a,* and pheophytin *a.* These were the three tetrapyrroles measured during the storage study. Due to solubility issues, the procedure was quite difficult. The procedure required massing a wide-mouthed bottle and stir bar and then adding an initial ~50 g acetone to ensure no bulk chlorophyll adhered to the bottle bottom. The bulk chlorophyll oil was then added drop-wise using a Pasteur pipette. This was problematic due to the low surface tension of

the oil and the rapidly evaporating acetone. The reported concentration of chlorophyll in the bulk oil was 16 percent, yielding a theoretical concentration of 230 ppm chlorophyll (by weight). The acetone was at 22 ^{o}C and the procedure was done under low light conditions. After massing out the acetone and chlorophyll, the bottle was sealed to prevent evaporation and agitated with the stir bar. The chlorophyll stock was prepared individually for each replication.

Photoreactor Set Up. A Thermo Oriel Photoreactor (Model 66902 Universal Arc Lamp Housing, Model 66910 Power Supply, Thermo Oriel Instruments, Stratford, CT) provided the high intensity light source used for this accelerated study (Figure 1). A 350 watt mercury lamp was focused on an optical cell through a cylindrical housing made of several components. In turn, the light passed through a focusing lens, an infrared filter, and a shutter housing. The infrared filter removed light in the infrared wavelengths without interfering with the visual and UV spectrum of the lamp. A quick-change filter holder contained a bandpass filter (model 10BPF25-XXX where XXX = 450, 550 or 650 nm were the center wavelength; Newport, Irvine, CA), which only allowed select wavelength regions to pass through; an alternative condition used no bandpass filter (full spectrum treatment). Bandpass width region was expressed using the full-width-half-max (FWHM) method. For each filter the FWHM was 25 nm, meaning that the transmission 12.5 nm to either side of the center wavelength is half the value of the maximum transmission. Please see Figure 2 for the actual transmission profiles of the bandpass filters used in this study. The light beam entered through a 1 inch diameter hole cut in a light-tight insulated box, and was focused on the optical cell containing the sample. The optical cell was held within a hollowed out aluminum block through which cold

antifreeze was circulated to control sample temperature over the exposure duration. This was referred to as the thermostatic block. The cooling bath and antifreeze circulator was turned on 20 minutes before starting a run. The optical cell (Thermo Fisher Scientific, Pittsburgh, PA, model 14-385-932E) was constructed of near-UV glass with a 50 mm path length and an internal volume of 16 mL; sample within the cell was agitated by a stir bar and contained by two plastic stoppers. Because the optical glass was not transparent at wavelengths below 320 nm, riboflavin was not impacted by light below this wavelength cut off. The TiO_2 film (contained within modified slide holders for ease of handling) sat ~0.5 cm in front of the optical cell. To aid in handling, the TiO_2 films were mounted in a 2x2" projector slide holder. A radiant power energy meter (Newport, Irvine, CA; model 70260) was permanently attached to the back of the box and was used to monitor light beam intensity after it had passed through the optical cell. The mercury lamp was turned on 10 minutes before starting a run in order to allow the light beam intensity to stabilize. The energy reaching the detector for the full spectrum, 450, 550, and 650 nm spectrum treatments in the absence of any film were 118 ±17.3, 2.84 ±0.416, 3.36 ±0.710, and 0.553 ± 0.246 mW respectively at the start of the run and decreased to 117 ±22.5, 2.21 ±0.465, 3.05 ±0.420, and 0.152 ± 0.057 mW at the end of the run.

Sample Exposure and Collection during the Photoreactor Experiment.

Riboflavin solutions (30 ppm) were exposed to light of four bandwidths (full spectrum, 450nm, 550 nm, 650 nm) with polyethylene films of three thicknesses (50, 75, 100 um) and five TiO_2 loads (0.5, 1.0, 2.0, 4.0, 8.0 wt%), placed immediately before the sample cell. Each treatment combination was tested, and with the inclusion the four controls (each of the bandwidths without a polyethylene film), a total of 64 exposure runs were

required. Due to time limitations replications were not performed. The optical cell, containing 12.1 mL of riboflavin solution, was exposed in the photoreactor over a period of 8 hours. The mercury lamp was warmed up for approximately 10 minutes prior to initiating sample exposure. Samples (100 uL) for HPLC analysis were taken at 0, 0.75, 1.5, 3.5, 6, and 8 hours of exposure. To obtain a sample, the shutter was used to temporarily block the light emitted from the Hg bulb. The optical cell was then removed from the holding cell within the thermostatic block and a 100 uL aliquot was pipetted into an HPLC vial (8x40 mm Total Recovery vials model 186000837c, Waters Corp, Milford, MA). The total period of unexposure to the Hg lamp was 1 minute during each sampling operation.

Sample temperature was monitored during three runs with an ECD Model 5100 Data LoggerLab (Milwaukie, OR) with a K-type thermocouple inserted through a rubber septum. The average sample temperature at the start of a run was $11.4 \pm 0.9\ ^{o}C$. The heat from the lamp increased the sample temperature to a maximum of $15.5 \pm 0.3\ ^{o}C$, which occurred approximately 15 minutes after the start of the run. At 0.75, 1.5, and 3.5 hour sampling times, the average temperatures were 10.7 ± 0.4, 4.3 ± 1.6, and $4.1 \pm 1.2\ ^{o}C$ respectively. The eventual steady state temperature of the sample was $\sim 4\ ^{o}C$.

Sample Preparation During the Storage Study Experiment. Photooxidation was monitored in four experimental mixtures (EM) during this study. Riboflavin was included in three EM during the study: riboflavin in phosphate buffer (RP), riboflavin in acetone & phosphate buffer (RAP), and riboflavin & tetrapyrroles in acetone & phosphate buffer (RCAP). The fourth EM was composed of tetrapyrroles in acetone & phosphate buffer (CAP). When riboflavin or tetrapyrroles were present in an EM their

targets concentrations were 30 ppm and 18 ppm (by mg/L), respectively. Immediately prior to each replication, 360 mL of each EM was prepared simultaneously. Containers were covered in foil and preparations occurred under low light conditions. The main component of all of the EMs was 324 mL of cold (5 ^{o}C) 1.1x phosphate buffer (containing 33.3 ppm dissolved riboflavin as appropriate), which comprised 90 percent by volume of the mixture. 36 mL of either ddH_2O or acetone was added as appropriate. If the EM contained tetrapyrroles (RCAP or CAP), the acetone used was the tetrapyrrole stock prepared earlier. The ddH_2O or acetone (with or without the tetrapyrroless) were both at room temperature (22 ^{o}C) and added to cold (5 ^{o}C) 1.1x phosphate buffer (with or without riboflavin). Stir bars blended the EMs as 20 mL aliquots were pippetted into exposure vials and time zero (T_o) samples were taken. One set of time zero samples for each of the four EM comprised a sample destined for riboflavin analysis and another destined for tetrapyrrole analysis. The exposure vials (Thermo Fisher Scientific, Pittsburgh, PA; Cat # 03-391-7F B7800-5; clear glass; type I class A; 23x85 mm; 24 mL capacity) were sealed with Teflon® caps (Thermo Fisher Scientific, Pittsburgh, PA; Cat # 03-391-7F; PTFE-Lined solid storage caps 20-400 finish) and covered with foil as appropriate. Vials were cooled in the dark for 20 minutes at 5 ^{o}C before being transferred to the walk-in refrigerator containing the fluorescent lighting setup.

Sample Exposure and Collection During the Storage Study Experiment.

Vials of each EM (RP, RAP, RCAP, CAP) were placed under fluorescent lighting. The caps of vials to be exposed to the light were labeled with the treatment name (RP, RAP, RCAP, CAP) while those that were to be light protected were covered with aluminum foil and then labeled with the treatment name and an additional “f” (RPf, RAPf, RCAPf,

CAPf). Vials were randomly arranged under 4 fluorescent lights (Westpointe Cool White 48" 34 Watt, 2 bulbs per fixture Chicago, IL) in the walk-in refrigerator (Tonka, Hopkins, MN) set to 5 ^{o}C. Light intensity ranged from 2020-1690 lux, measured at the level of the vials using an Extech light meter (Extech Instrument Corp., Walthaun, MA) before and after each replication.

Baseline, or time zero (T_o), samples for all EM were analyzed by HPLC for both riboflavin and tetrapyrroles Subsequent sampling (T_1-T_7) for riboflavin was only done on samples containing riboflavin (RP, RPf, RAP, RAPf, RCAP, RCAPf). Likewise, subsequent sampling for tetrapyrroles was only done on treatments that initially contained tetrapyrroles (RCAP, RCAPf, CAP, CAPf).

Riboflavin Analysis for both the Photoreactor and Storage Experiments. Riboflavin concentration in the sample was analyzed before and after light exposure using a Waters (Milford, MA) 1525 HPLC with a Restek Ultra Aqueous C-18 column (100 x 3.2 mm, 3 um particle size, 100 angstrom pore size; Restek, Bellefonte, PA). One of two alternative guard columns were used (Restek Ultra Aqueous C8 (preliminary work and initial runs)) or C18 (10 x 2.1 mm; majority of the analyses). The solvent system was an isocratic mixture of 84% (v/v) solvent A (1% (v/v) glacial acetic acid in H_2O) and 16% solvent B (acetonitrile). Both solvents were HPLC or analytical grade. The column temperature was 25 °C and the flow rate was 0.5 mL/min. Each run lasted 10 minutes. The injection volume, using a Waters 717plus autosampler (Milford, MA), was initially 20 µL but was subsequently reduced to 5 µL. A Waters 2487 absorbance detector was used to monitor the absorbance at 270 nm during the HPLC runs.

The riboflavin peak eluted at approximately 5 minutes (roughly 4 times the void elution time) and adequate separation was achieved. Riboflavin was quantified using external standards (1, 6, 10, 20, 30 ppm riboflavin) to create a standard curve from the peak area counts. The peak area counts of the samples were then compared to the standard curve to quantify the amount of photosensitizer remaining. During the photoreactor study most samples and standards were analyzed using duplicate injections in an effort to reduce random variability. During the storage study, samples were analyzed once due to time constraints; duplicate injections of select standards were done to ensure random variability in riboflavin peak area was low (approx. <2%) within a run.

Tetrapyrrole Analysis. The fluorescence of pyropheophytin *a,* pheophytin *a,* and chlorophyll *a* were analyzed before and after light exposure using an Agilent (Santa Clara, CA) 1260 Infinity HPLC system with a Restek Ultra Aqueous C-18 column (100 x 3.2 mm, 3 um particle size, 100 angstrom pore size; Restek, Bellefonte, PA). Retention times for chlorophyll *a,* pheophytin *a,* and pyropheophytin *a* were 10.2, 23.1, and 30.6 minutes, respectively. The relative retention times were 10.4, 24.7, and 33.0, repectively. The void volume was 0.45 mL. A Restek Ultra Aqueous C18 (10 x 2.1 mm) guard columns was used. The solvent system was an isocratic mixture of 50% (v/v) solvent A (2:1 (v:v) Methanol:H_2O) and 50% solvent B (ethyl acetate). Both solvents were HPLC or analytical grade. The column temperature was 25 °C and the flow rate was 0.5 mL/min. Each run lasted 35 minutes. The injection volume was 25 uL using an Agilent 1260 autosampler (model G1329A; Santa Clara, CA), the temperature of the sample compartment was maintained at 5 ^{o}C. An Agilent 1260 Diode Array Detector (model G1321B; Santa Clara, CA) was used to report the absorbance at 410 and 430 nm during

the HPLC runs. This same diode array detector continuously recorded the absorbance spectrum between 300-750 nm throughout each run. Fluorescence, instead of absorbance, was the primary method used to enumerate chlorophyll *a,* pyropheophytin *a,* and pheophytin *a* because of the increased specificity and sensitivity. The fluorescence detector was an Agilent 1260 Infinity FLD (model G1321B; Santa Clara, CA) and excitation and emission parameters were 430 nm and 657 nm, respectively.

Data Manipulation for the Photoreactor Experiment. Comparison of film efficiency in protection of riboflavin then was estimated using a single point. The first order reaction coefficient for each film was found by graphing the natural log of riboflavin concentration (in mole/L) vs. time and taking the slope of the linear regression line. Only the first three sampling points (0, 0.75, and 1.5 hrs) were used to determine the slope because riboflavin concentration often dropped below the level of detection before the 4^{th} sampling point (3.5 hrs) while under full spectrum light. The same method of analysis was used in the 450, 550, and 650 nm conditions. The full spectrum 0.5% TiO_2 films and control (no film) could not be analyzed in this manner because the limits of riboflavin detection were reached before the 1.5 hr sampling occurred. The control and theoretical ideal points were included on the graphs and in the regression analysis when possible.

Statistical Analysis for the Photoreactor Experiment. The first order reaction coefficients (as described above) were analyzed using a full factorial ANOVA. The main effects were film thickness (50, 75, 100 um), film TiO_2 load (0.5, 1.0, 2.0, 4.0, 8.0%), and spectrum (450 nm and full spectrum). The main effects and interactions were evaluated using an α of 5%. The full spectrum 0.5% TiO_2 films and control (no film) could not be

included because the limits of riboflavin detection were reached before the 1.5 hr sampling occurred. Data from the 550 and 650 nm conditions were also excluded. This decision was made because the observed variations in apparent effectiveness were suspected to be due to the limits of detection in concentration and experimental error. No intercept term was included in the model used in the ANOVA. JMP 9.0.0 Statistical Discovery Software (SAS Institute Inc. 2010) was used to conduct this analysis. This study was performed without replications. Duplicate measures were taken for each condition.

Data Manipulation for the Storage Study. Apparent first order reaction coefficients for riboflavin and tetrapyrroles for each treatment were found using the same method as during the photoreactor experiment. The only differences in methodology were the number of sampling points used in the calculations. The first six sampling points (0, 1, 2, 4, 8, and 16 hours) were used to determine the slope of riboflavin degradation. Apparent first order reaction coefficients for pyropheophytin *a* and pheophytin *a* used the 0, 1, 2, and 8 hour time points. The 4 hour time point was excluded from the analyses because data for one of the two RCAPf replications at 4 hours was missing. Chlorophyll *a* coefficients were calculated using the 0, 1, and 2 hour time points because the concentration dropped below the limit of detection before the 8 hour sample was taken. Tetrapyrrole calculations were done using peak area counts while riboflavin calculations were done using molarity.

As will be discussed later, the degradation observed may not be truly a first order reaction. The value of this analysis is that it models the relationship between the data from multiple time points during a replication accurately.

Statistical Analysis for the Storage Study. The first order reaction coefficients for each treatment (found as described above) were analyzed for statistical significance ($p<0.05$) using a one-way ANOVA. Least significant means analysis using the Student's t test was used to compare the means. JMP 9.0.0 Statistical Discovery Software (SAS Institute Inc. 2010) was used to conduct this analysis.

RESULTS AND DISCUSSION

Wavelength Effects. Increased riboflavin protection generally was seen with both increasing TiO_2 loads and with increasing film thicknesses when riboflavin was exposed to a broad spectrum of light (Figure 3). The titanium dioxide load of a film had more impact on protection of riboflavin than did film thickness. It should be noted however that TiO_2 load increased by a factor of 16, while thickness only increased by a factor of 2. TiO_2 load, increasing from 0.5 wt% to 8 wt%, correlates with greater retention of riboflavin after exposure up to 8 hrs to the full spectrum of the Hg lamp. The 8 wt% TiO_2 load films provided the most riboflavin protection followed by the 4 wt% load films and so on. Increasing film thickness also increased the protection afforded to riboflavin, but did not have as much influence as TiO_2 load.

In the absence of any TiO_2 film or wavelength filter, the initial 30 ppm riboflavin was reduced to zero (or near zero) ppm in less than 45 minutes. As can be seen in Figure 3, the most effective films (8 wt% TiO_2 load) extended the time required for this to occur to 6-8 hours. The effectiveness of the least opaque films (0.5 wt% TiO_2 50 um and 0.5 wt% TiO_2 100 um) could not be compared to the control because the riboflavin concentration was reduced to zero very rapidly.

Figure 4 plots the experimentally observed first order reaction coefficient for riboflavin degradation under full spectrum light versus opacity. The resulting R^2 of the linear regression was 0.831 and $p_{value}<0.0001$ by the Pearson Correlation test illustrated the strong correlation between film opacity and riboflavin protection under full spectrum conditions.

Riboflavin degraded in the narrow wavelength region surrounding 450nm, which occurred extensively when no film was used as a barrier but was retarded when TiO_2-containing films intercepted these wavelengths. As can be seen in Figure 5, increased riboflavin protection was seen with both increasing TiO_2 loads and film thicknesses. As observed in the full spectrum treatment, the TiO_2 load of a film had more impact on its protective ability than did its thickness. Figure 6 plots an experimentally observed first order reaction coefficient for riboflavin degradation versus opacity for the 450 nm bandpass filter exposures. The resulting R^2 of the linear regression was 0.783 and $p_{value}<0.0001$ by the Pearson Correlation test illustrated the strong correlation between film opacity and riboflavin protection found here.

The longer wavelength ranges (550 and 650 nm filters) did not greatly affect riboflavin concentration. As discussed elsewhere, the light intensity of each of the treatments differed from each other, and light intensity is also an important factor in photooxidation. As can be seen in Figure 7, little to no degradation of riboflavin was seen, even after 22 hours, under the 550 nm and 650 nm control conditions. The observed variation in apparent effectiveness of the films coupled with the 550 nm or 650 nm (see Figures 8, 9, 10, and 11) bandpass filters is probably due to limits of detection in concentration as well as experimental error. No correlation between film properties and

riboflavin protection was observed because the bandpass filters were already blocking the damaging wavelengths of light (R^2 = 0.085 (550 nm) and 0.026 (650 nm), Figures 10 and 11). Pearson Correlation p_{values} were 0.2736 and 0.5489, respectively.

Given that the broad spectrum light treatment provided all riboflavin absorption wavelengths (370, 400, 450, 570) above 320 nm, it was not surprising that this treatment had the fastest rate of riboflavin degradation *(8,9)*. Degradation due to photooxidation can proceed using Type I or Type II mechanisms, with promotion of the photosensitizer to an excited state by a photon of appropriate energy (and therefore λ) followed by the transfer of energy from the photosensitizer to another molecule *(8,9,13)*. The selected bandpass filters included the visible wavelengths (450 and 570 nm) at which riboflavin and chlorophyll, another photosensitizer, absorb light so degradation of riboflavin over time was expected in the 450 nm and 550 nm bandpass ranges *(3,9)*. Degradation of riboflavin within the 550nm bandpass filter range did not occur as expected. This may be attributed to transmission window of the filter, which did not have as much transmission at 570 nm (2%), compared to the 450 bandpass filter, which had a maximum transmission (54%) at the wavelength, 450 nm, most strongly associated with riboflavin absorption in the visible spectrum. Secondly, while 570 nm is a minor absorption band for riboflavin at a pH of 7, it is not clear if 570 nm is still an absorption band in the phosphate buffer system used in this experiment. The 650 nm bandpass filter was anticipated to serve as a control, demonstrating that riboflavin degradation would be minimal.

Statistical Analysis. Table 1 shows the unbalanced ANOVA analysis conducted as described in the methods selection. Notably, the model does not include an intercept term or data from the 550 and 650 nm bandpass filter combinations. The main effects:

TiO_2 load, thickness, and spectrum were significant at or below the $p<0.05$ level. This indicates that changes in each of these effects had a significant influence on the rate of riboflavin degradation. The only interaction that was found to be significant was the TiO_2 Load•Spectrum term, indicating strong evidence ($p=0.0240$) that the influence of one of these effects varied as the other effect changed. Depending upon the properties of the TiO_2 film in question, the relative transmission of light wavelengths will often differ *(14)*. This is a likely explanation for the observed interaction between TiO_2 load and spectrum. The other interactions tested in this ANOVA were not significant ($p>0.05$).

Table 1. Full Factorial ANOVA output for observed first-order rate constants of riboflavin decay, based on 0, 0.75, and 1.5 hr samples. Output includes degrees of freedom, F-value, and p-value (Pr>F) for the main effects: film TiO_2 load (0.5, 1.0, 2.0, 4.0, 8.0%), film thickness (50, 75, 100 um), Spectrum (450 nm and full spectrum). Two and three-way interactions are also included. Model does not include an intercept term.

Source	**Degrees of Freedom**	**F-value**	**Pr > F**
TiO_2 Load	1	8.7631	0.0075*
Thickness	1	60.9126	<.0001*
TiO_2 Load•Thickness	1	0.1012	0.7535
Spectrum	1	50.4830	<.0001*
TiO_2 Load•Spectrum	1	5.9226	0.0240*
Thickness•Spectrum	1	1.2436	0.2774
TiO_2 Load•Thickness•Spectrum	1	3.4714	0.0765

* Indicates significance at the $p<0.05$ level

As discussed earlier, ground state riboflavin can absorb photons of specific wavelengths and then undergo intersystem crossing to triplet riboflavin *(15–17)*.

$$^1\text{Rib} + \gamma \rightarrow {}^1\text{Rib}^* \rightarrow {}^3\text{Rib}^*$$

Photooxidation then proceeds forward by a Type I or Type II mechanism, eventually passing the energy of the photon off to other photo-labile compounds and regenerating ^{1}Rib *(8,9,18)*. In the model system used in this study, the only photo-labile compound is riboflavin and, therefore, most of the energy absorbed during the photooxidative process is used to degrade riboflavin. This is in contrast to dairy products that have a great

number of compounds that can be oxidized instead of riboflavin *(8,10,19,20)*. Compounds present in milk that are particularly sensitive to oxidation due to riboflavin are unsaturated lipids and whey proteins *(8,10,19,20)*.

The ratio of Type I reactions to Type II photoreactions will differ depending upon the relative concentrations of oxygen and potential substrates *(13,20)*. Type I reactions yield a radical species ($O_2^{\bullet-}$), while Type II reactions yield singlet oxygen ($^1O_2$) *(8,9,13)*. The Type I mechanism should dominate in milk due to high concentration of substrates *(13,20)*. In this study's model system, the percentage of Type II reactions dominate due to the decreased availability of substrates. In addition, the amount of oxygen present in the system was not controlled during the study. Taking a sample involved removal of the cylindrical cell's cap, so each time a sample was taken there was an opportunity for the headspace oxygen content to be replenished. If this significantly increased the $^3O_2$ (triplet oxygen) concentration this would also increase the rate of Type II reactions. The relative and absolute rates for each Type I and Type II reactions is known to change over time as the concentrations of oxygen and potential substrates change within a system *(13)*. The ratio of the two reaction types is important in foods due to the kinds of off-flavors produced and the kinds of food components (protein or lipids) oxidized to produce those off-flavors *(8,13,20)*.

Riboflavin that is not associated with other milk components, known as free riboflavin, is more susceptible to photooxidation than riboflavin that is *(21)*. Riboflavin is present in both forms in milk, while only free riboflavin was used in the model system *(21)*.

The additional compounds found in milk also can affect the outcome of light exposure in a variety of ways. Bovine milk contains several components that have demonstrated antioxidant effects during photooxidation. The bovine whey proteins β-lactoglobulin and bovine serum albumin have both been confirmed to compete with oxygen to quench triplet riboflavin *(20)*. Ascorbic acid and α-tocopherol, naturally occurring antioxidants at the concentrations found in milk, also have been studied for their contribution to controlling photooxidation *(22)*. Milk also has compounds with pro-oxidant effects. Hematoporphyrin, protoporphyrin, and chlorophyll *a* & *b* have all been identified as known or probable photooxidants in cheese and/or milk *(1,2,5,12)*. The net effect of these compounds will vary depending upon many factors such as: dairy product, initial concentrations, packaging, and storage conditions.

It is important to note the experimental conditions of this study before extrapolating the results obtained here to milk stored under commercial conditions. The environment surrounding riboflavin, light intensity at each wavelength, and surface area to volume ratio all differ when compared to fluid milk as typically purchased by the consumer. Milk is a more opaque liquid than the model system used in this study. For example, the effective penetration depth for 405 nm wavelength light in whole milk has been determined to be only 0.9 cm and 2.4 cm for 578 nm light *(23)*. Only in this region will photooxidation occur, which is why, all other things being equal, the photo-protection of single-serving milk containers is of greater concern than 1 gallon packages. In contrast, the riboflavin in phosphate buffer solution was a clear-pale yellow color (which was easy to see-through) before turning colorless as the riboflavin degraded.

The 350 watt mercury lamp light source used was far more intense than conventional dairy case lighting and had a different spectral distribution as well. According to the manufacturer the mercury lamp has a potential flux of ~18,000 lumens *(24)*. Assuming 1/4 of that flux is concentrated in an area the size of the cylindrical cell (~3 cm^2) results in an illuminance of 10^7 lx. A study examining 8 dairy cases reported lighting values ranging from 750-6460 lx, leading the authors to use 2000 lx as an approximate median value in their future work *(25)*. While the photoreactor is certainly more intense than dairy case lighting more research must be done before quantitative comparisons can be made. As Mortensen and others state (2004) "Unfortunately, measurement of total light intensity cannot be used for prediction of damage potential, unless the light sources measured have identical spectral distribution of radiation;…" *(26)*. The storage study detailed in the next section allowed for a quantitative comparison of the two lighting methods under very specific circumstances.

Storage Study

Results. As expected fluorescent light exposure led to decreases in the concentration of photolabile compounds. Concentrations of all four compounds of interest (riboflavin, chlorophyll *a*, pyropheophytin *a*, pheophytin *a*) were found to decrease in the presence of light (Figures 12, 13, 14, 15). Foil wrapped treatments had little to no decrease in the concentration of these same compounds. The presence of tetrapyrroles was found to increase the rate of riboflavin loss, while the presence of riboflavin was found to decrease the rate of tetrapyrrole loss. A comparison of Figures 12-15 shows that riboflavin degraded at a slower rate than the tetrapyrroles in all of the

EM. The concentration of riboflavin in all light exposed treatments approached zero after 16-48^{+} hours of light exposure. The concentration of pyropheophytin *a* and pheophytin *a* approached zero after 8-16 hours of light exposure in all treatments. Chlorophyll *a* concentrations approached zero after 4-8 hours of light exposure in all treatments.

Table 2. Observed first order rate constants (hr^{-1}) for riboflavin and associated R^2 values for each treatment containing riboflavin during the storage study.

Treatment*	**x hr^{-1} (x± sd)**	**R^2**
RP	-0.0422^{b} ± 0.0055	0.99588
RAP	-0.0626^{c} ± 0.0012	0.98836
RCAP	-0.1037^{d} ± 0.0015	0.96711
RPf	-0.0017^{a} ± 0.0009	0.31269
RAPf	-0.0028^{a} ± 0.0050	0.10680
RCAPf	0.0002^{a} ± 0.0003	0.02977

$^{a, b, c, d}$Means followed by the same letter are not significantly different (p=0.05) using Student's t LSD test.
*Treatments were riboflavin in phosphate buffer (RP), riboflavin in phosphate buffer and acetone (RAP), and riboflavin and tetrapyrroles in phosphate buffer and acetone (RCAP). Each light exposed treatment had a foil wrapped light protected control indicated with an "f" appended to the code (ex. RPf).

Quantitative Comparison of Fluorescent Lighting and Photoreactor. The observed first-order rate constant for riboflavin degradation in the RP treatment was -0.0422 ± 0.0055 hr^{-1} (avg ± std dev) (Table 2). This reaction rate is much slower than those that could be computed for the TiO_2 films in combination with the photoreactor's full spectrum 350 W Hg arc lamp, which ranged in value from -2.49 to -0.345 hr^{-1} (Figure 4). In the absence of any TiO_2 film or wavelength filter, the initial 30 ppm riboflavin was reduced to zero (or near zero) ppm before the second sampling point (0.75 hrs) during the photoreactor experiment. Later work with the photoreactor using smaller sampling intervals determined the first order rate constant for the no-film no-wavelength filter (control) to be -12 ± 3 hr^{-1}. Under the specific conditions of the experiments

conducted, the photoreactor degrades riboflavin on the order of 300 times more quickly than fluorescent lighting. Again, it must be emphasized that this estimate only applies to the solution parameters, containers, volumes, lighting conditions, temperatures, and other factors outlined earlier. One solution parameter that should be able to vary without changing the estimate is riboflavin concentration, provided, of course, that the concentration does not rise high enough to significantly reduce the light penetration through the cylindrical cell or vial.

Due to the differing spectral distributions of the light sources, the addition of compounds (such as tetrapyrroles) with different absorption characteristics could likely result in ratios other than 300:1. Photoreactor runs using the model system(s) tested during the storage study (RAP, RCAP, CAP) could be used to gather the necessary information needed to calculate the correct ratio(s).

Effect of Tetrapyrroless on Riboflavin Oxidation. As can be seen in Figure 12, riboflavin degradation among light exposed treatments, in the order of fastest to slowest, were RCAP, RAP, and finally RP. Significant ($p<0.05$) differences in the apparent first order rate constants were found between these three light exposed treatments (Table 2). During the preparations for this study, the addition of acetone (10% by volume) at the level necessary to introduce significant amounts of nonpolar tetrapyrroles into the aqueous system was found to affect the rate of riboflavin degradation. The exact mechanism for this is yet to be determined. Acetone has been reported to be a photosensitizer under certain conditions *(27–33)*. Reconciling this phenomenon with how commonly acetone is used in lab work without concern about these effects is difficult. The solvent effects of acetone on riboflavin in this system might also be

responsible for the increase in photooxidation observed during this study. While a direct comparison between the RP and RCAP treatments cannot be done, RAP and RCAP can be compared. Addition of the tetrapyrroles (composed mostly of pyropheophytin *a* and pheophytin *a*) in the amount present in the RCAP treatment significantly increased the degradation of riboflavin when compared to the RAP treatment ($p<0.05$) as can be seen in Table 2.

The mechanism by which the riboflavin degradation rate increased in the presence of tetrapyrroles has not been definitively determined by this experiment.

Table 3. Observed first order rate constants (hr^{-1}) and associated R^2 values for pyropheophytin *a*, pheophytin *a*, and chlorophyll *a* for each treatment containing tetrapyrroles during the storage study.

	Pyropheophytin *a*		**Pheophytin *a***		**Chlorophyll *a***	
Treatment*	**hr^{-1} (x± sd)**	**R^2**	**hr^{-1} (x± sd)**	**R^2**	**hr^{-1} (x± sd)**	**R^2**
RCAP	$-0.335^{b} \pm 0.013$	0.959	$-0.302^{b} \pm 0.014$	0.961	$-0.189^{b} \pm 0.040$	0.976
CAP	$-0.500^{c} \pm 0.043$	0.959	$-0.470^{c} \pm 0.043$	0.959	$-0.171^{b} \pm 0.084$	0.954
RCAPf	$-0.013^{a} \pm 0.002$	0.971	$-0.014^{a} \pm 0.002$	0.990	$-0.006^{a} \pm 0.052$	0.834
CAPf	$-0.010^{a} \pm 0.002$	0.967	$-0.011^{a} \pm 0.000$	0.969	$0.023^{a} \pm 0.006$	0.458

[a, b, c]Means followed by the same letter within a column are not significantly different ($p=0.05$) using Student's t LSD test.
*Treatments were riboflavin and tetrapyrroles in phosphate buffer and acetone (RCAP), and tetrapyrroles in phosphate buffer and acetone (CAP). Each light exposed treatment had a foil wrapped light protected control indicated with an "f" appended to the code (ex. CAPf).

Effect of Riboflavin on Select Tetrapyrroles. As can be seen in Figures 13-15 degradation of the measured tetrapyrroles was faster for the CAP treatment than the RCAP treatment. Significant ($p<0.05$) differences in the apparent first order rate constants were found between these light exposed treatments for pyropheophytin *a* and pheophytin *a* (Table 3). Significant ($p<0.05$) differences in the apparent first order rate

constants were not found between chlorophyll *a* concentrations for these light exposed treatments (Table 3). Addition of riboflavin to the tetrapyrroles appears to slow their degradation in the model system used. This finding is in agreement with the findings (discussed above) that riboflavin degradation rate increased in the presence of tetrapyrroles.

CONCLUSIONS

TiO_2 load within polyethylene films of varying thicknesses can be designed to optimize for riboflavin protection from damaging UV and visible wavelengths, as demonstrated in this model system. Understanding the relationship of film opacity, based on TiO_2 load and film thickness, in controlling riboflavin excitation provides guidance in developing materials that provide optimum protection for this nutrient, and may protect milk and dairy product flavor. As the dairy industry's knowledge of the interactions between all of the photosensitizers in milk in cheese grows, so will their ability to provide quality products to consumers through cost-effective packaging. The development of model systems and methods of accelerating light-based storage studies will help food scientists and their partners in industry gain this knowledge.

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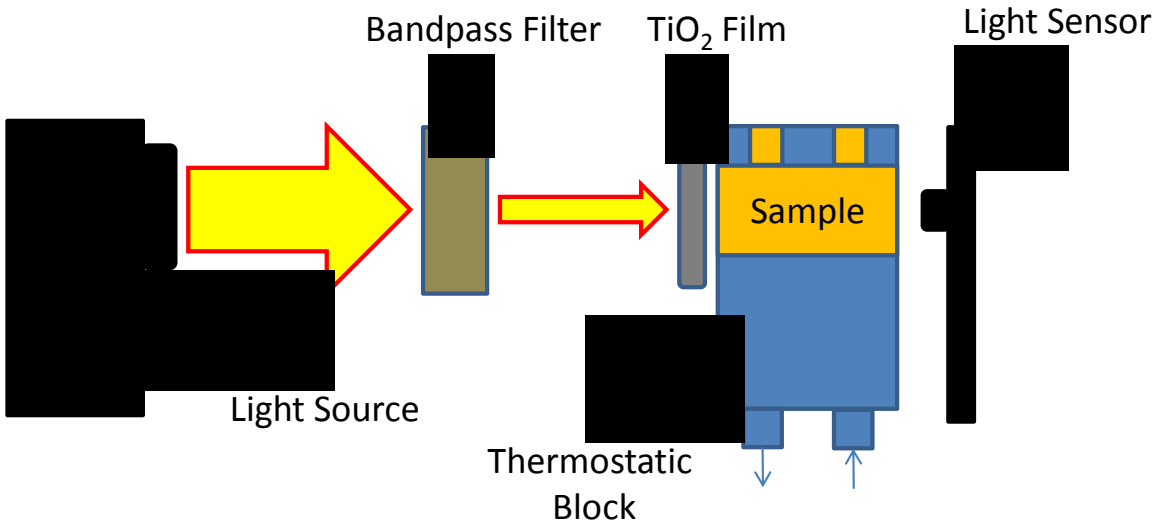

Figure 1. Simplified diagram of Thermo Oriel Photoreactor setup. Light from a 350 watt mercury arc lamp traveled through bandpass filters and/or TiO_2 films before reaching the optical sample cell.

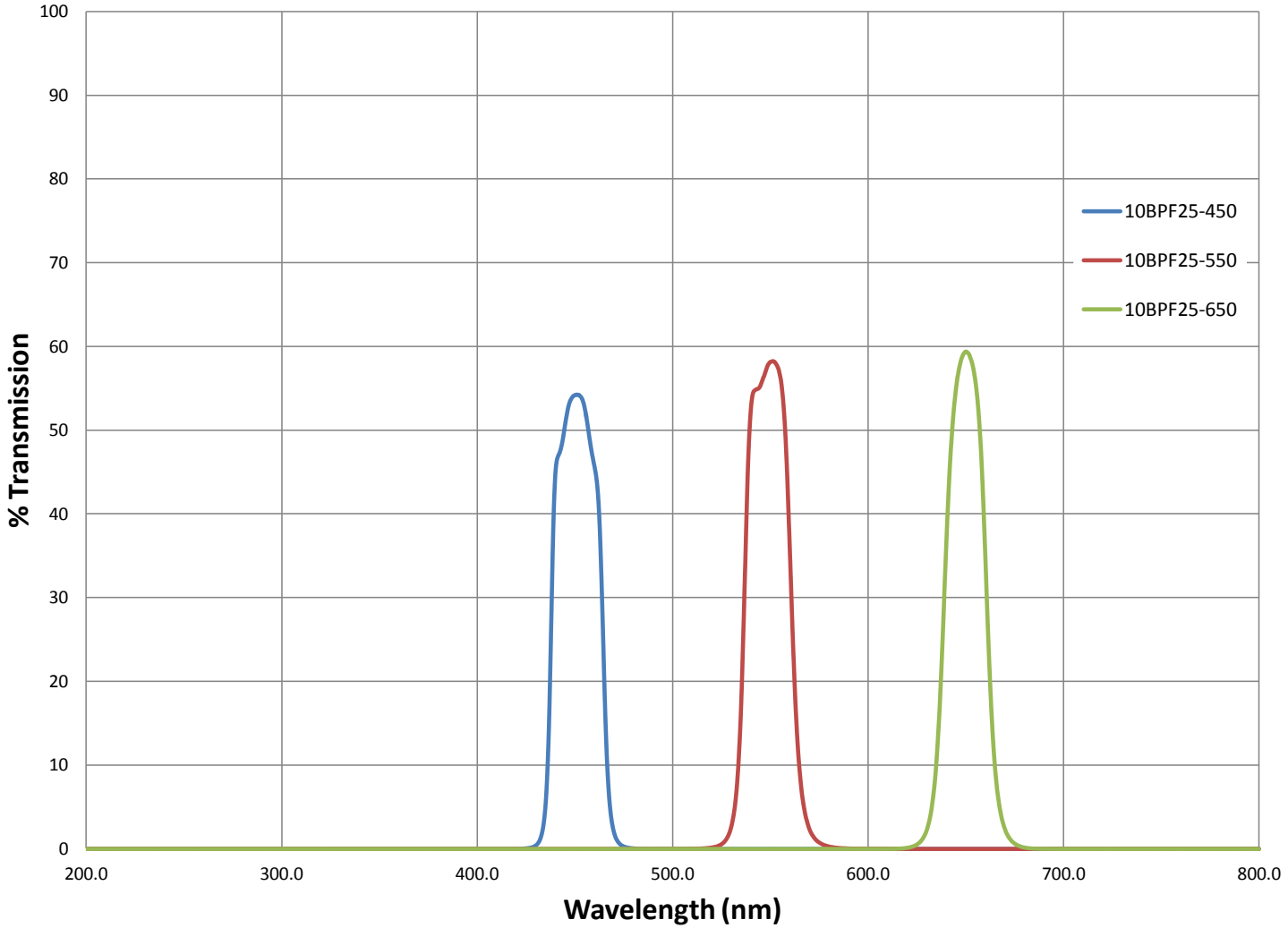

Figure 2. Wavelengths and % transmission for Newport (Irvine, CA) bandpass filters used. Transmission measured using a Shimadzu UV-2550 UV-vis spectrophotometer (Kyoto, Japan).

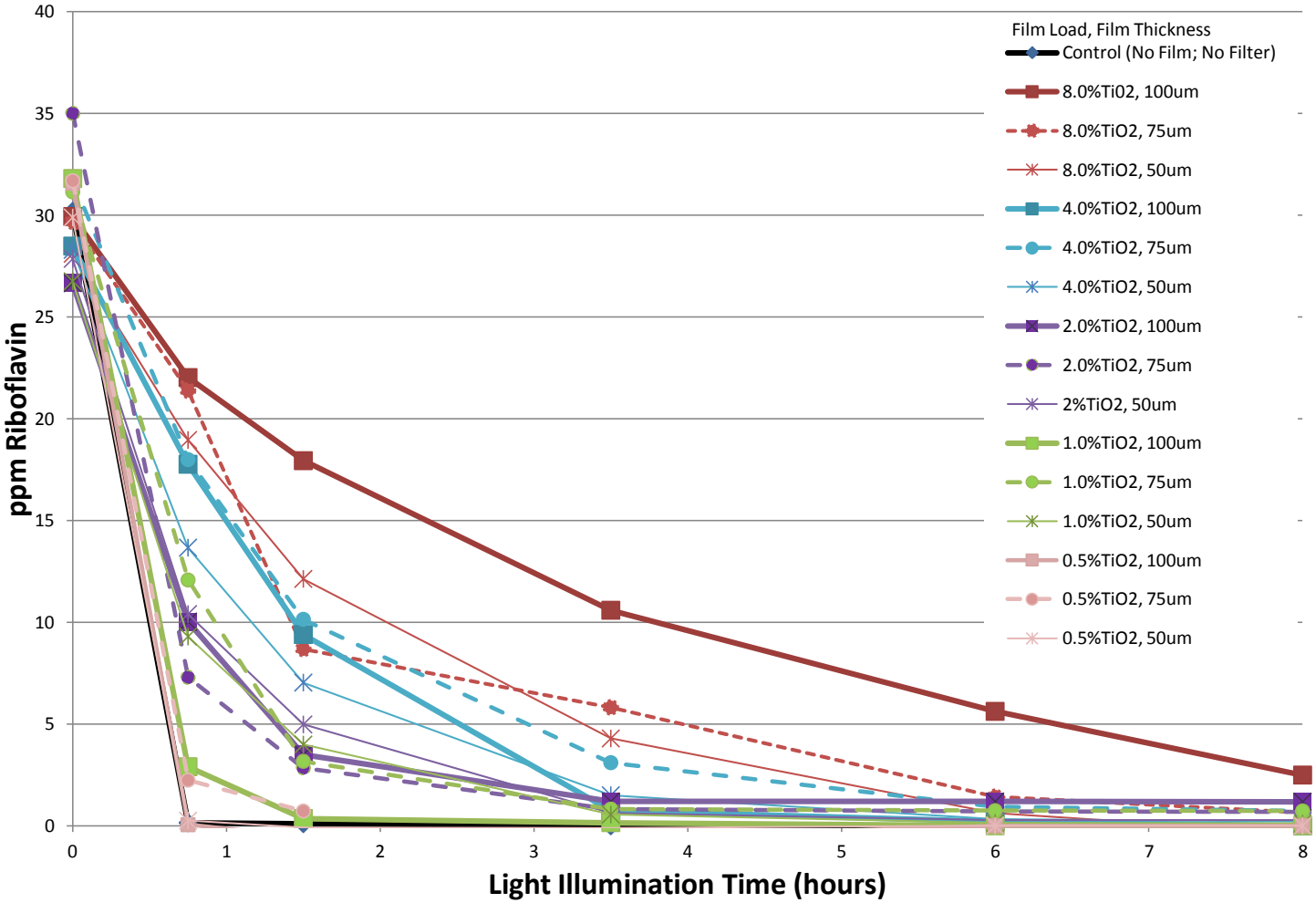

Figure 3. Riboflavin concentration over time in phosphate buffer at pH 6.5 for TiO_2 films in combination with full spectrum light from a 350 watt Hg arc lamp.

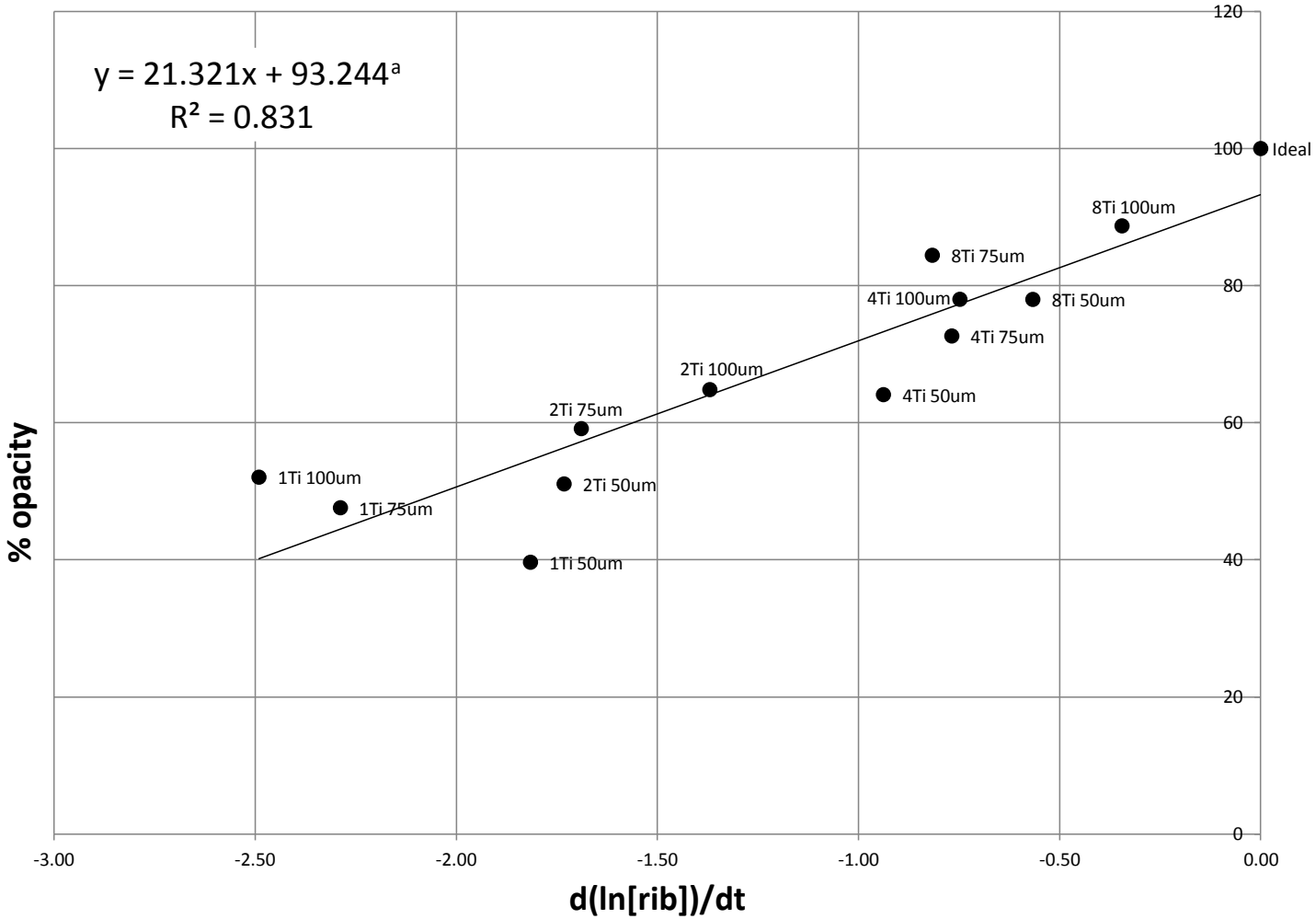

Figure 4. Linear regression of % opacity and observed first-order rate constants for riboflavin degradation in phosphate buffer at pH 6.5 for TiO_2 films in combination with full spectrum light from a 350 watt Hg arc lamp. [a]Regression equation includes a theoretical film that prevents any riboflavin degradation (Ideal).

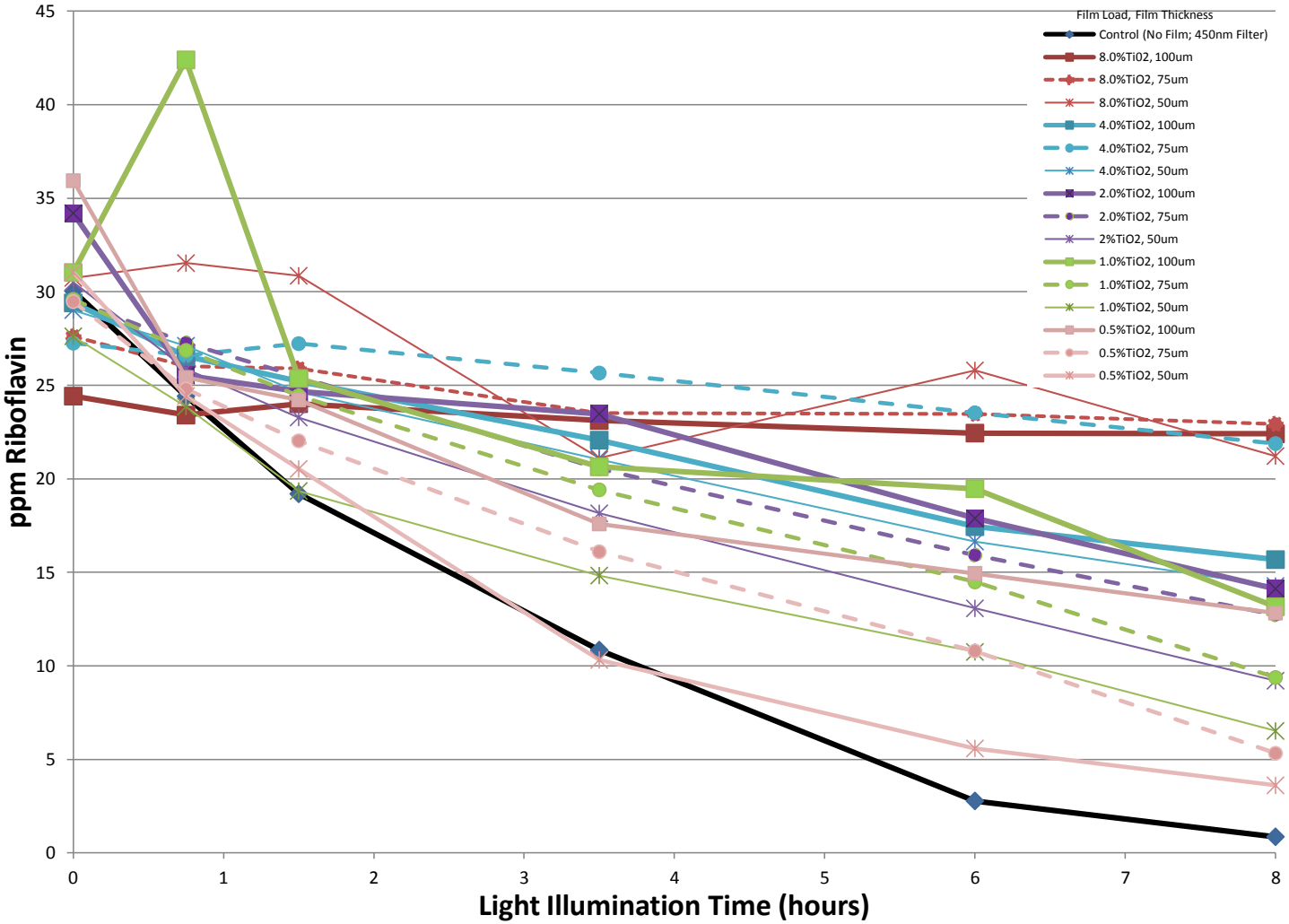

Figure 5. Riboflavin concentration over time in phosphate buffer at pH 6.5 for TiO_2 films in combination with the 450 nm bandpass filter conditions.

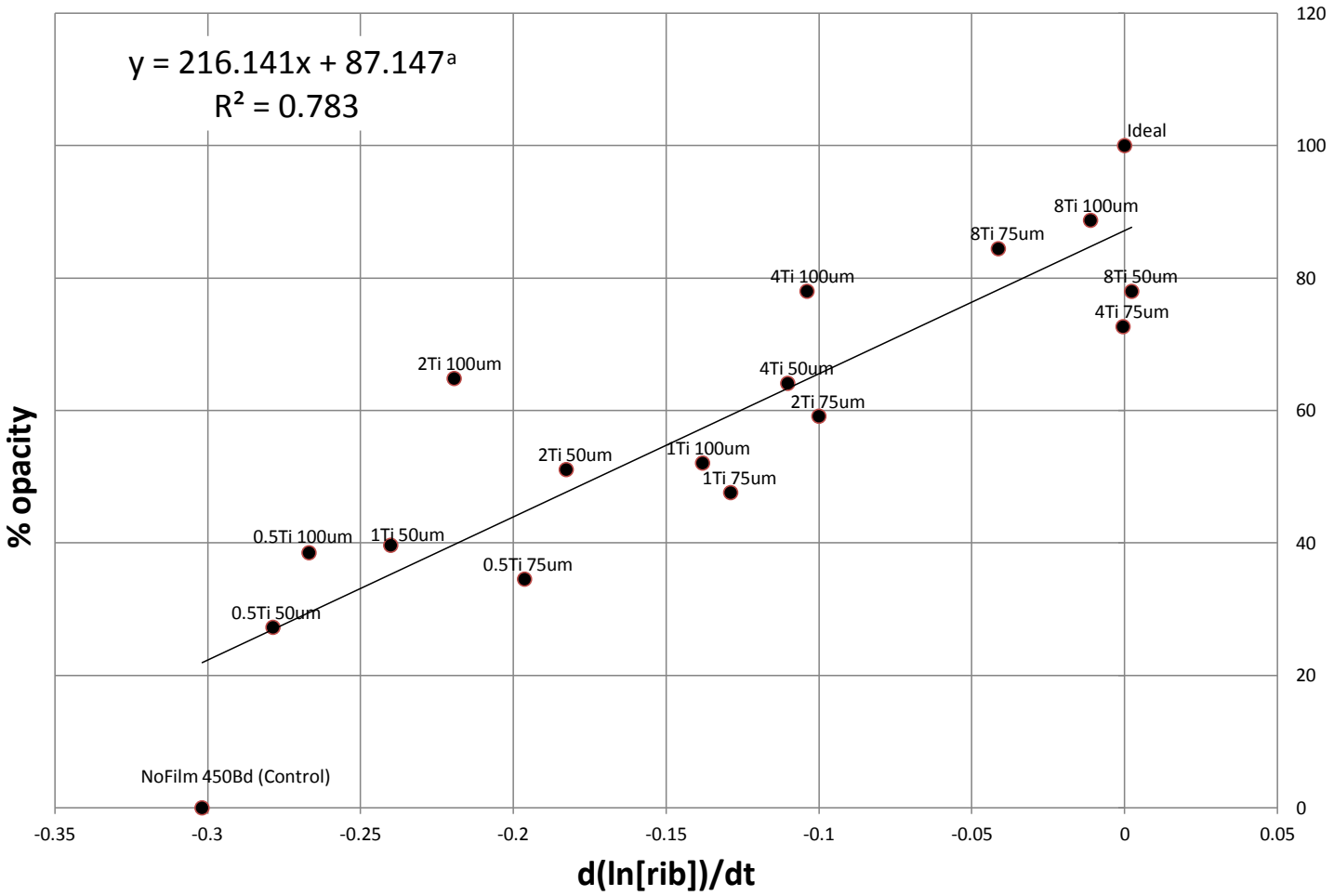

Figure 6 Linear regression of % opacity and observed first-order rate constants for riboflavin degradation in phosphate buffer at pH 6.5 for TiO_2 films in combination with the 450 nm bandpass filter conditions. [a]Regression equation includes control (NoFilm 450 Bd Control) and a theoretical film that prevents any riboflavin degradation (Ideal).

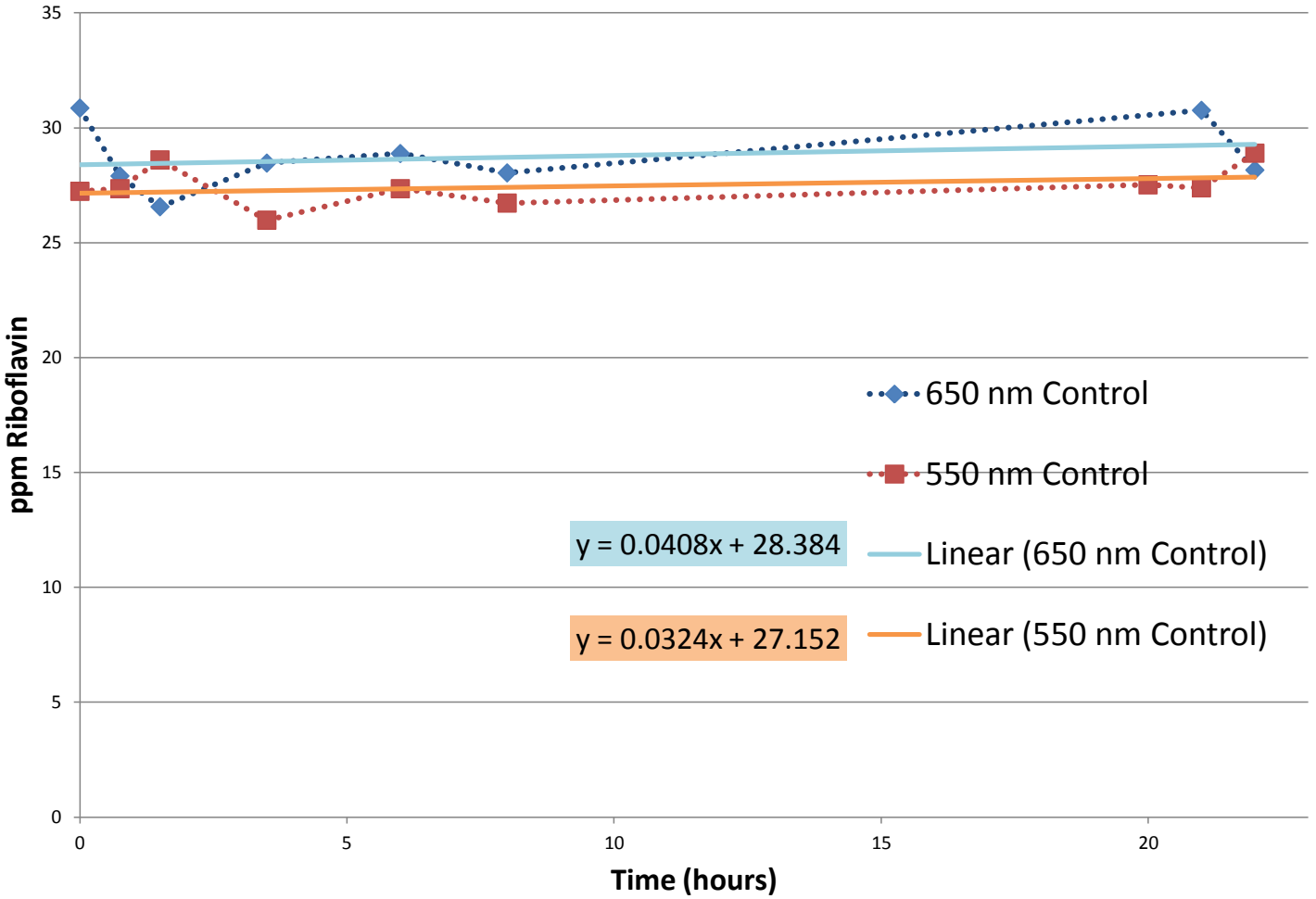

Figure 7. Riboflavin concentration over 22 hours in phosphate buffer at pH 6.5 for the 650 and 550 nm bandpass filters using no TiO_2 films (the controls).

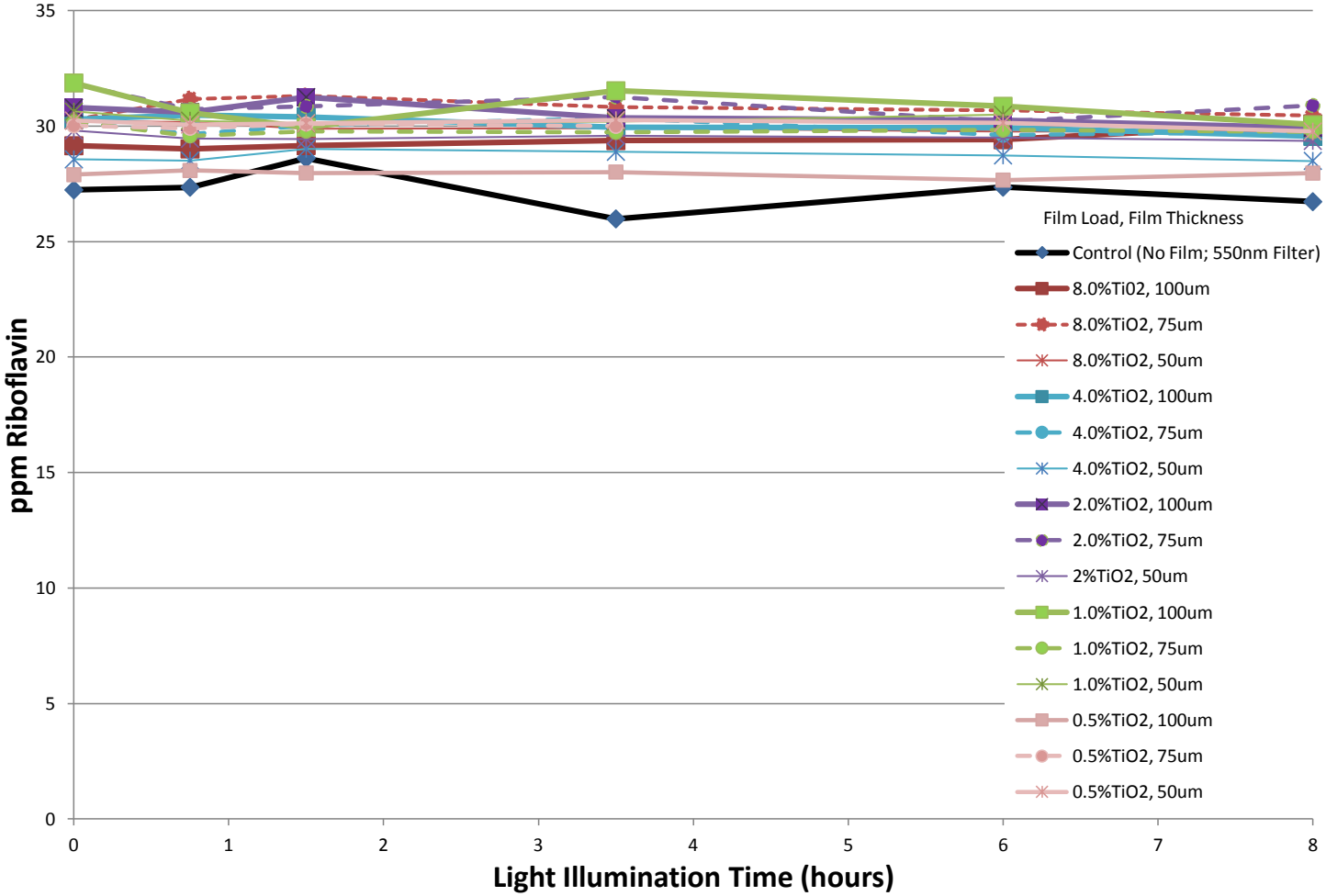

Figure 8. Riboflavin concentration over time in phosphate buffer at pH 6.5 for TiO_2 films in combination with the 550 nm bandpass filter conditions.

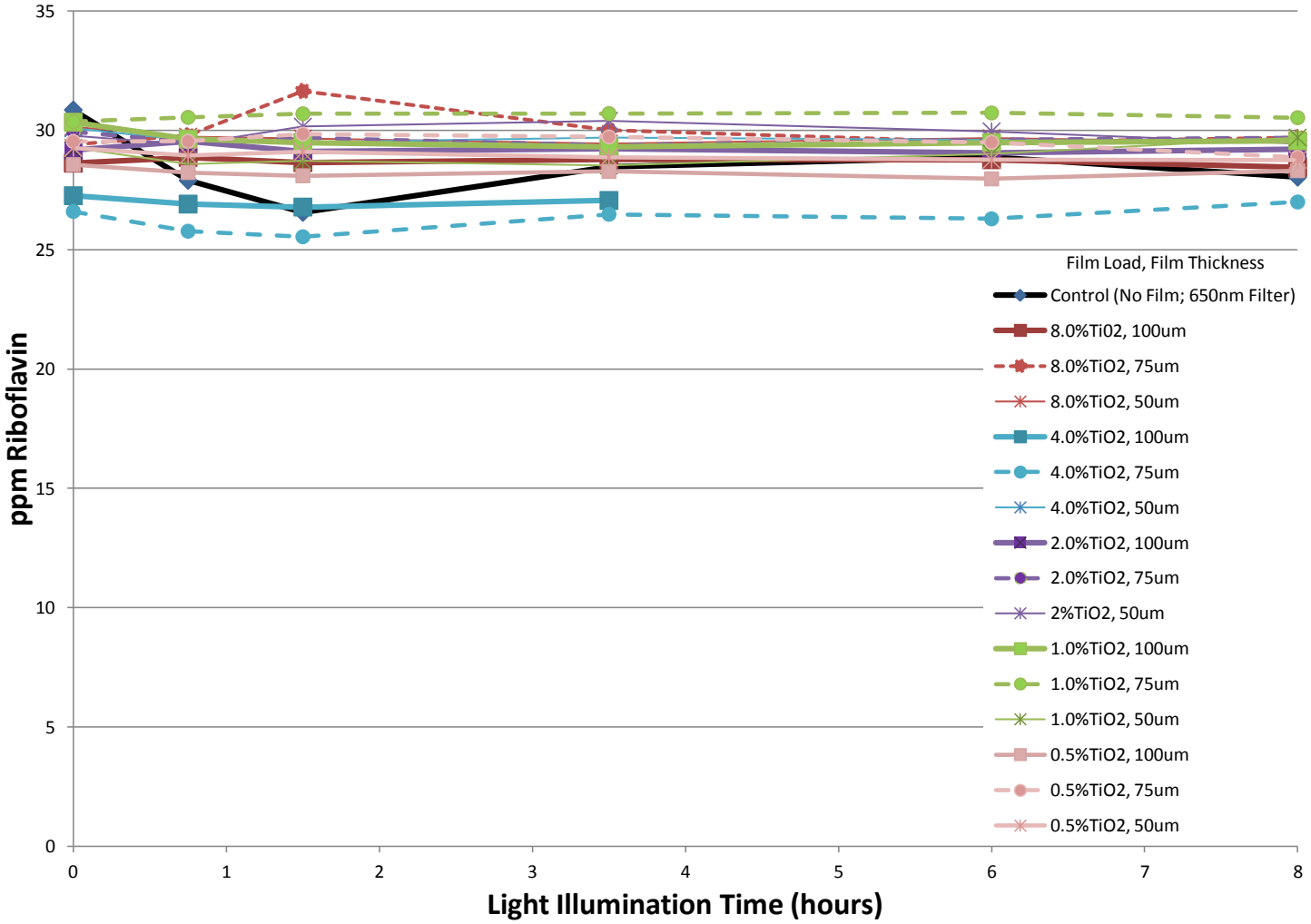

Figure 9. Riboflavin concentration over time in phosphate buffer at pH 6.5 for TiO_2 films in combination with the 650 nm bandpass filter conditions.

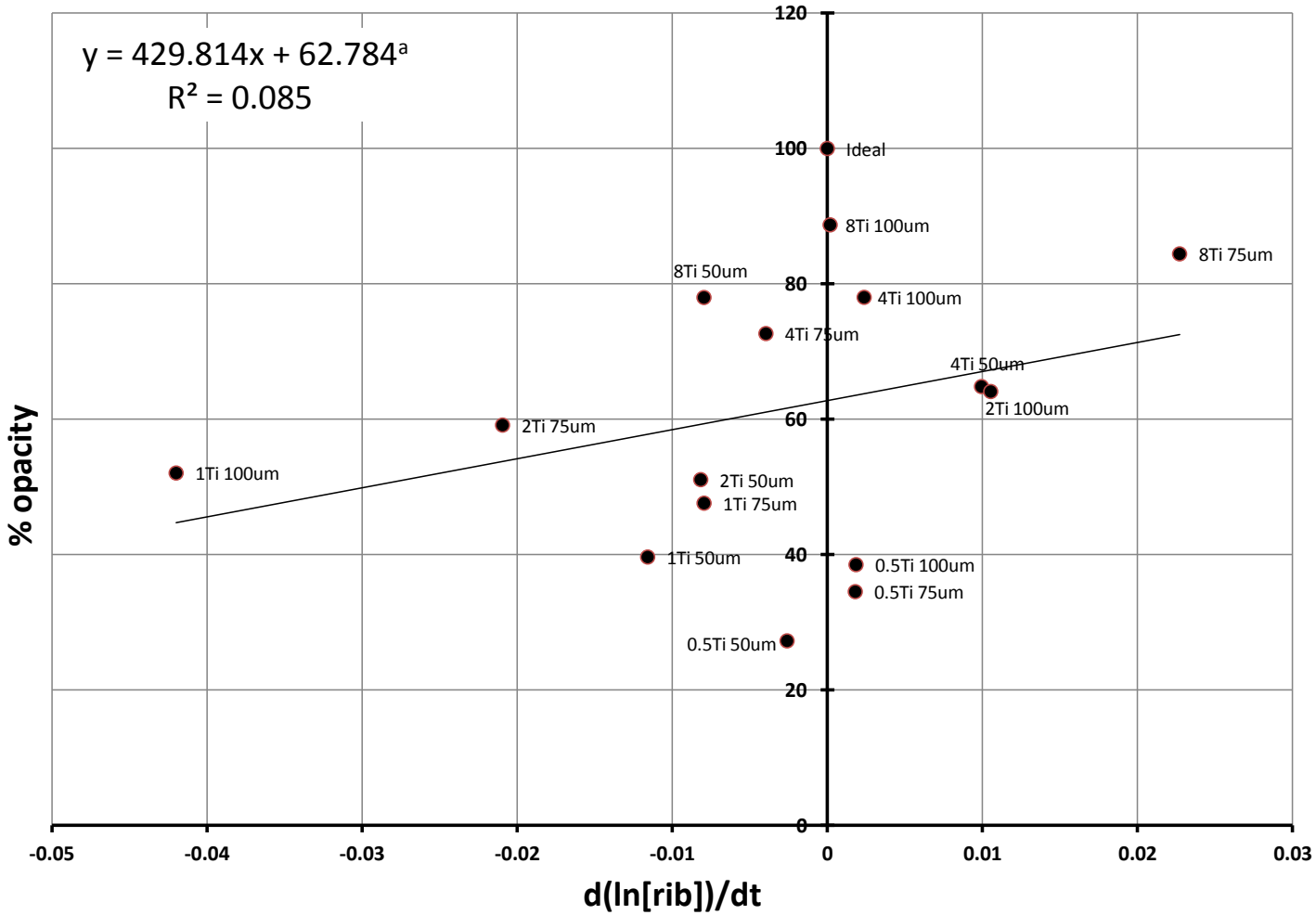

Figure 10. Linear regression of % opacity and observed first-order rate constants for riboflavin degradation in phosphate buffer at pH 6.5 for TiO_2 films in combination with the 550 nm bandpass filter conditions.
[a]Regression equation includes a theoretical film that prevents any riboflavin degradation (Ideal).

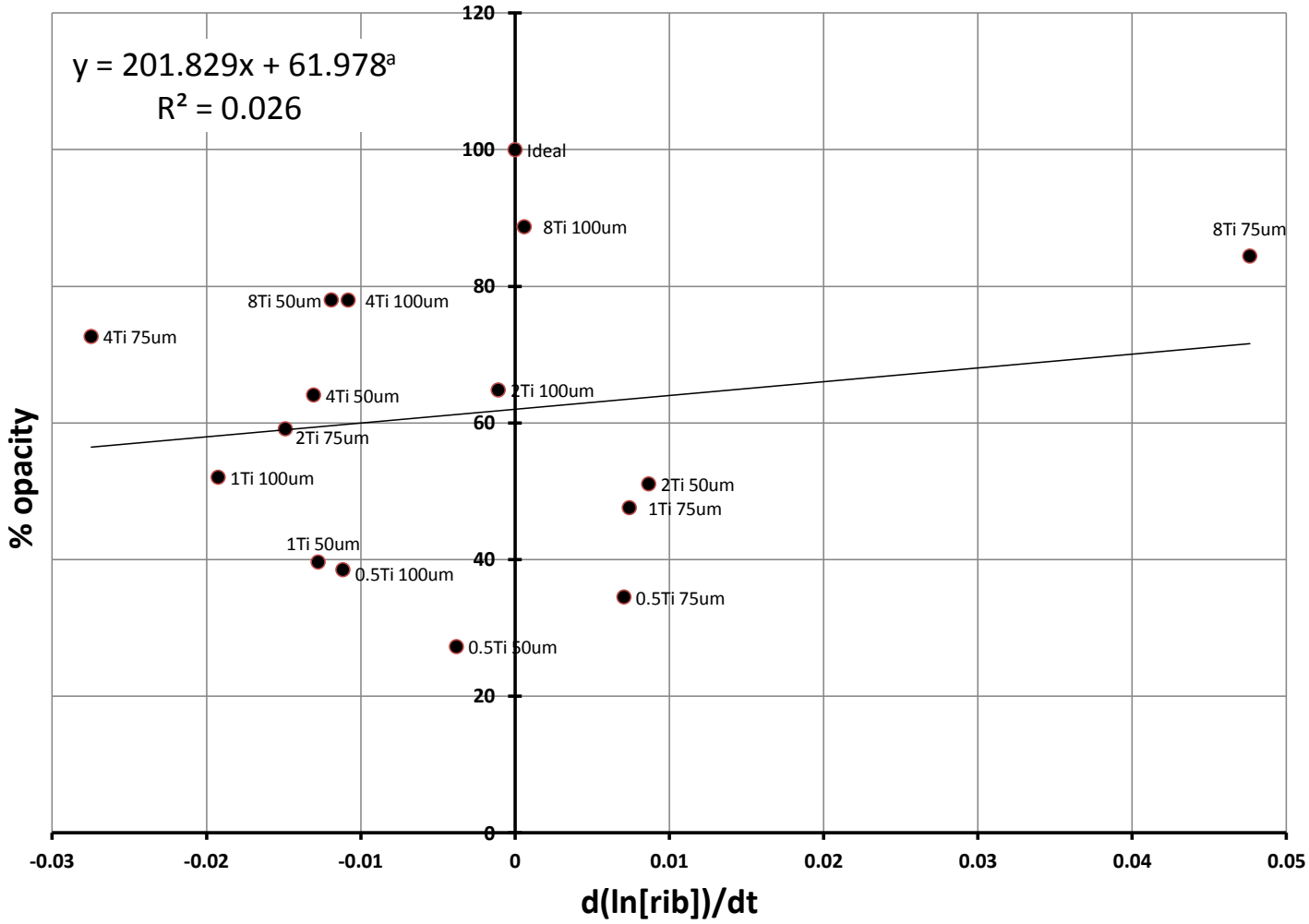

Figure 11. Linear regression of % opacity and observed first-order rate constants for riboflavin degradation in phosphate buffer at pH 6.5 for TiO_2 films in combination with the 650 nm bandpass filter conditions.
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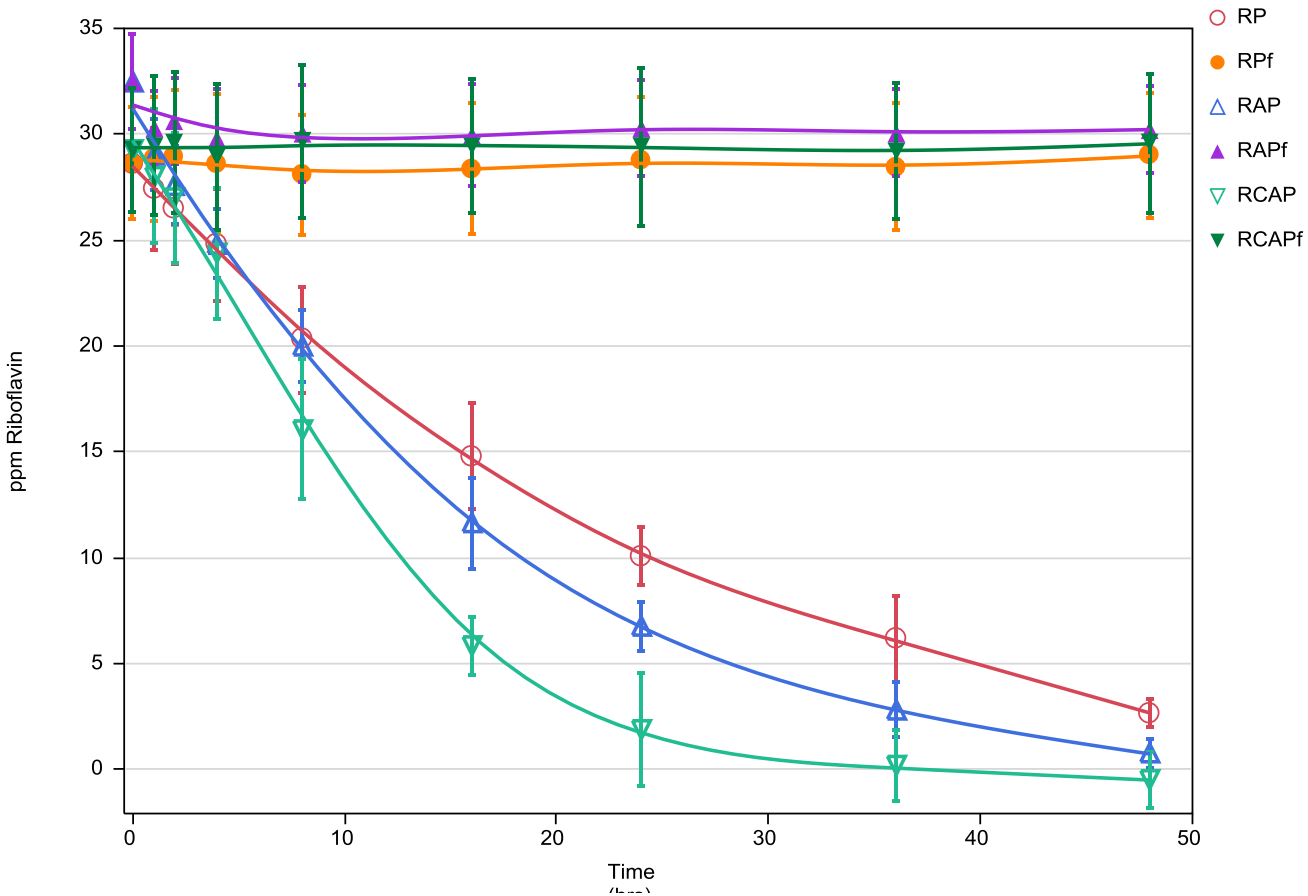

Figure 12. Riboflavin concentration ± standard deviation for samples held at 6 ± 1 ^{o}C after 0, 1, 2, 4, 8, 16, 24, 36, 48 hours of exposure to 2020-1690 lux fluorescent lighting. Treatments were riboflavin in phosphate buffer (RP), riboflavin in phosphate buffer and acetone (RAP), and riboflavin and tetrapyrroles in phosphate buffer and acetone (RCAP). Each light exposed treatment had a foil wrapped light protected control indicated with an "f" appended to the code (ex. RPf).

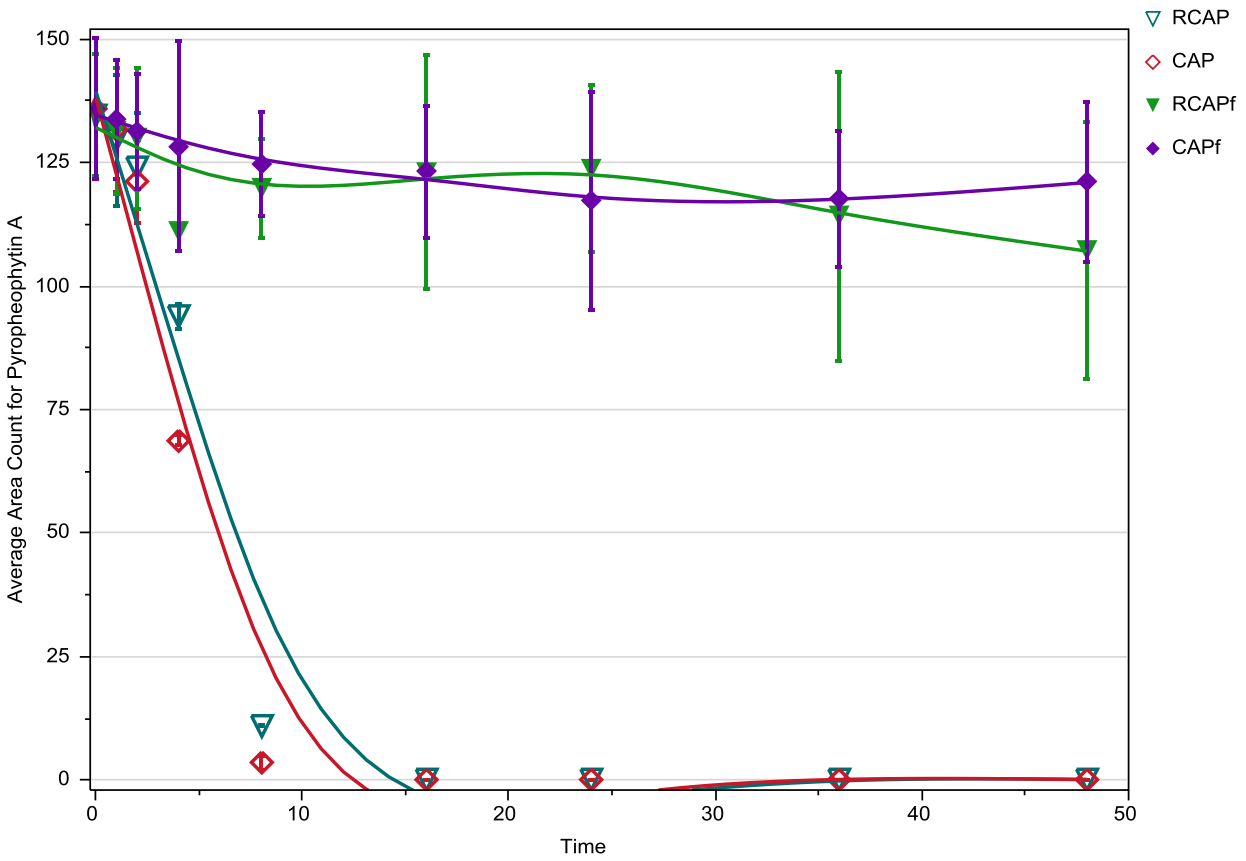

Figure 13. Pyropheophytin *a* average area counts ± standard deviation for samples held at 6 ± 1 ^{o}C after 0, 1, 2, 4, 8, 16, 24, 36, 48 hours of exposure to 2020-1690 lux fluorescent lighting. Treatments were riboflavin and tetrapyrroles in phosphate buffer and acetone (RCAP), and tetrapyrroles in phosphate buffer and acetone (CAP). Each light exposed treatment had a foil wrapped light protected control indicated with an "f" appended to the code (ex. CAPf).

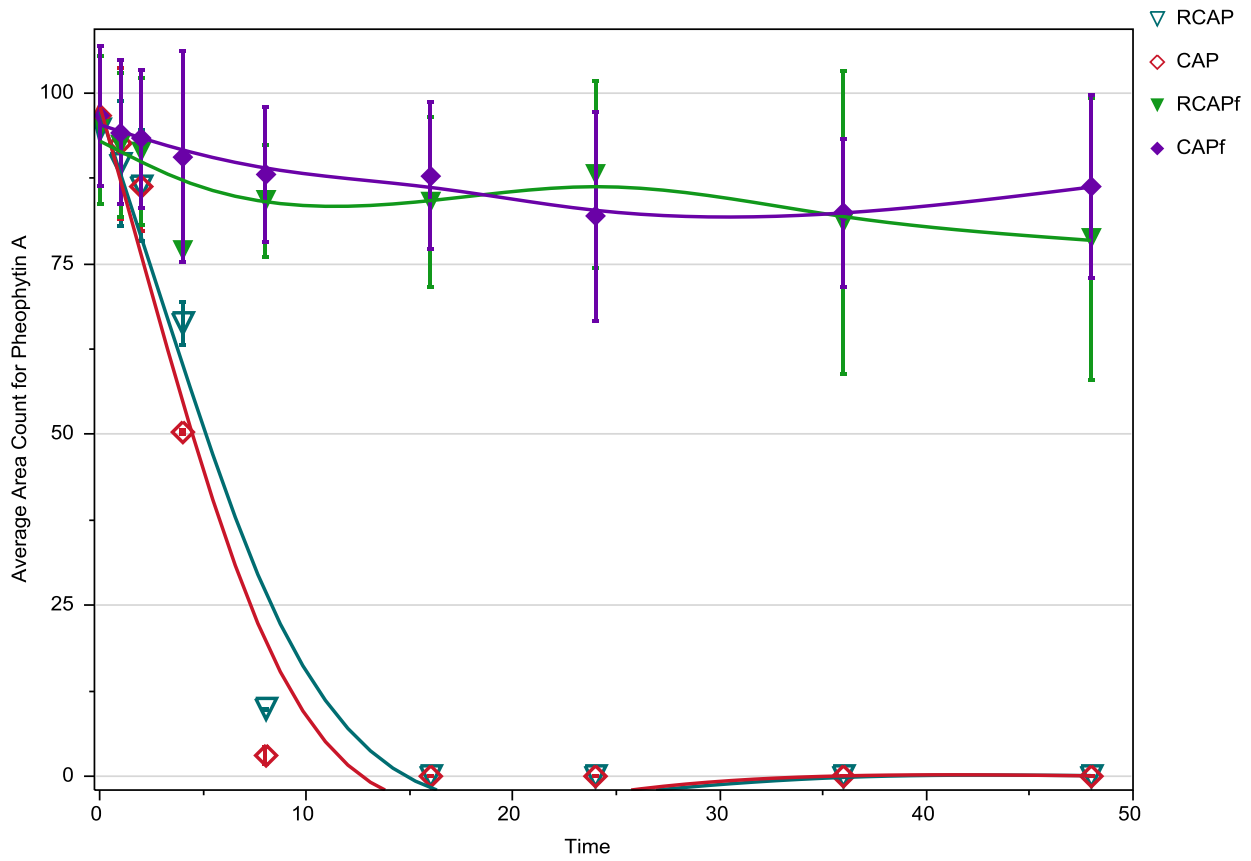

Figure 14. Pheophytin *a* average area counts ± standard deviation for samples held at 6 ± 1 ^{o}C after 0, 1, 2, 4, 8, 16, 24, 36, 48 hours of exposure to 2020-1690 lux fluorescent lighting. Treatments were riboflavin and tetrapyrroles in phosphate buffer and acetone (RCAP), and tetrapyrroles in phosphate buffer and acetone (CAP). Each light exposed treatment had a foil wrapped light protected control indicated with an "f" appended to the code (ex. CAPf).

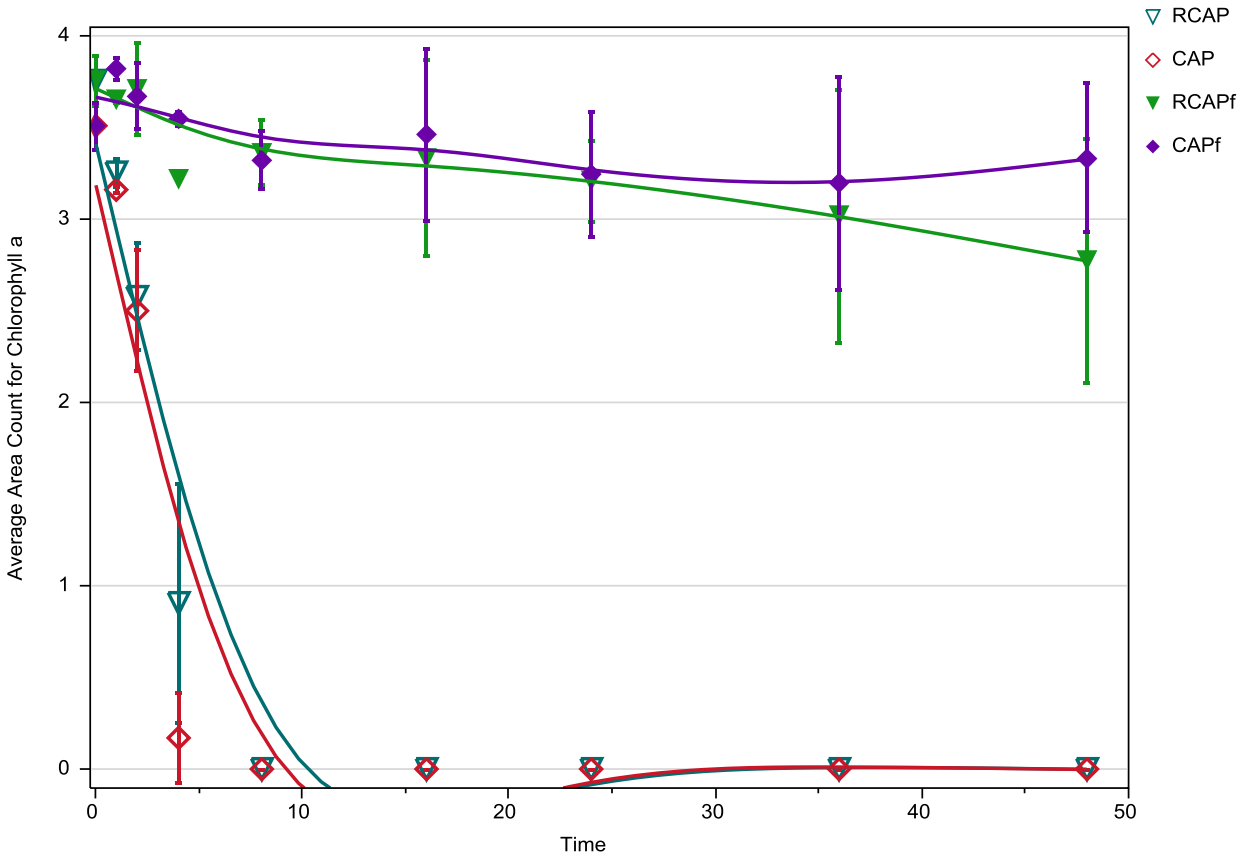

Figure 15. Chlorophyll *a* average area counts ± standard deviation for samples held at 6 ± 1 ^{o}C after 0, 1, 2, 4, 8, 16, 24, 36, 48 hours of exposure to 2020-1690 lux fluorescent lighting. Treatments were riboflavin and tetrapyrroles in phosphate buffer and acetone (RCAP), and tetrapyrroles in phosphate buffer and acetone (CAP). Each light exposed treatment had a foil wrapped light protected control indicated with an "f" appended to the code (ex. CAPf).

APPENDICES

Appendix A

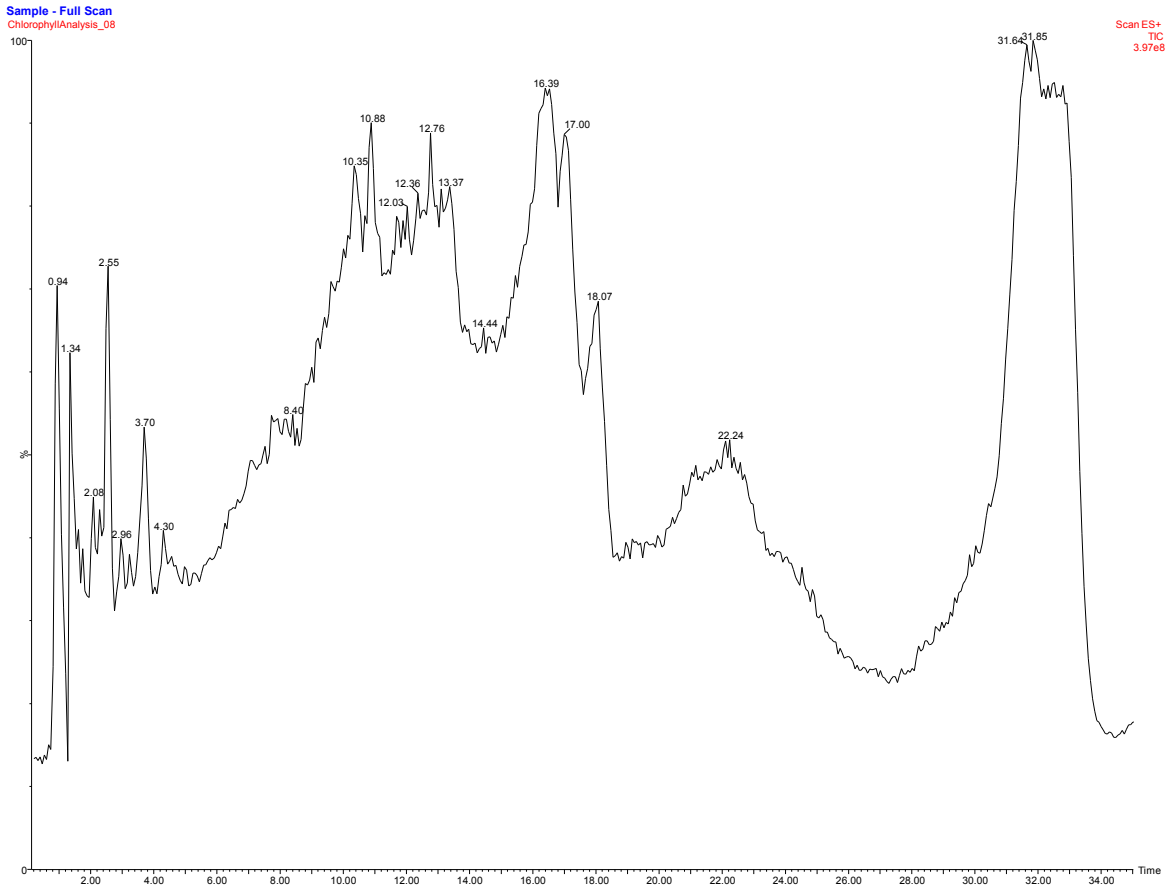

Figure A1. Full scan of chlorophyll soluble oil (MP Biolmedicals, Solon, OH; Cat # 206208, Lot# 6354K) using a Restek Ultra Aqueous C-18 column (100 x 3.2 mm, 3 um particle size, 100 angstrom pore size; Restek, Bellefonte, PA), an isocratic mixture of 50% (v/v) solvent A (2:1 (v:v) Methanol:H_2O) and 50% solvent B (ethyl acetate) at a total flow rate of 0.5 mL/min, and conditions outlined in Figure A2. UPLC separations were performed on a Waters Acquity H-class MS analysis was performed on a Waters Acquity TQD mass spectrometer equipped with a z-spray electrospray interface.

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Switch 4	No Change
Infusion	No Change
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Flow rate	5 µl/min
Reservoir	B
Refill	No Action
API Probe Delay Temp	20 °C
Run events	Yes
Event 1	0.10 min Refill Refill
Event 2	1.00 min Flow State Combined
Event 3	1.00 min Infusion Start
Number Of Functions	1

Function 1 : MS Scan, Time 0.00 to 35.00, Mass 500.00 to 1050.00 ES+

Type	MS Scan
Ion Mode	ES+
Data Format	Centroid
Start Mass	500.0
End Mass	1050.0
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Figure A2. LCMS settings used during the full scan illustrated in Figure A1.

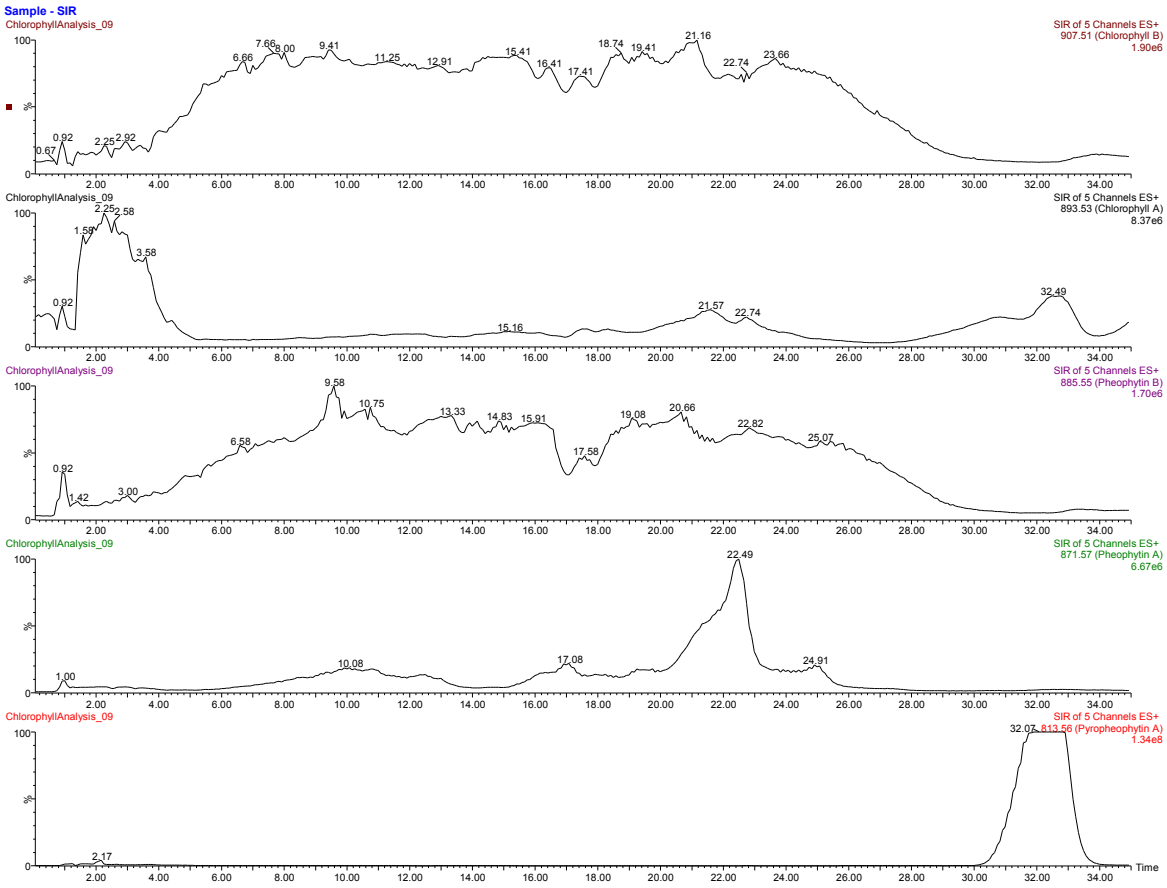

Figure A3. SIR scan of chlorophyll soluble oil (MP Biolmedicals, Solon, OH; Cat # 206208, Lot# 6354K) using a Restek Ultra Aqueous C-18 column (100 x 3.2 mm, 3 um particle size, 100 angstrom pore size; Restek, Bellefonte, PA), an isocratic mixture of 50% (v/v) solvent A (2:1 (v:v) Methanol:H_2O) and 50% solvent B (ethyl acetate) at a total flow rate of 0.5 mL/min, and conditions outlined in Figure A4. UPLC separations were performed on a Waters Acquity H-class MS analysis was performed on a Waters Acquity TQD mass spectrometer equipped with a z-spray electrospray interface.

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Switch 3	No Change
Switch 4	No Change
Infusion	No Change
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Flow rate	7 µl/min
Reservoir	B
Refill	No Action
API Probe Delay Temp	20 °C
Run events	Yes
Event 1	0.10 min Refill Refill
Event 2	1.00 min Flow State Combined
Event 3	1.00 min Infusion Start
Number Of Functions	1

Function 1 : SIR of 5 masses, Time 0.00 to 35.00, ES+

Type	SIR
Ion Mode	ES+
Data Format	Centroid
Scan Time (sec)	4.00
Span (Da)	0.00
Start Time (min)	0.0
End Time (min)	35.0

Ch	Mass(Da)	Dwell(s)	Cone(V)	Delay(s)	Compound
1	813.56	0.994	Tune	-1.000	Pyropheophytin A
2	871.57	0.994	Tune	-1.000	Pheophytin A
3	885.55	0.994	Tune	-1.000	Pheophytin B
4	893.53	0.994	Tune	-1.000	Chlorophyll A
5	907.51	0.994	Tune	-1.000	Chlorophyll B

Figure A4. LCMS settings used during the full scan illustrated in Figure A4.

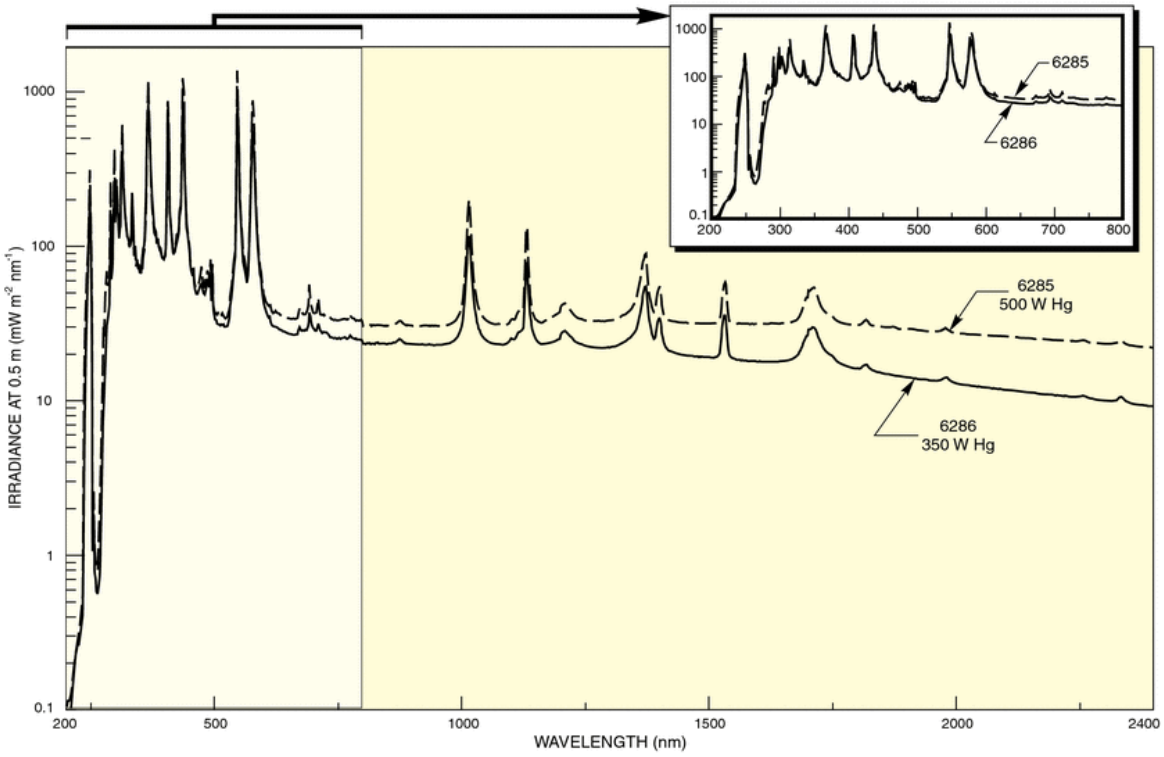

Figure A5. Spectrum of the 350 Watt Mercury Lamp used in the photoreactor. Newport Corp. *350 Watt Mercury Lamp.* Available at: http://search.newport.com/?q=*&x2=sku&q2=6286. Accessed 1 December 2011.